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Departamento de Química Física Aplicada

DIGESTIBILIDAD, ALERGENICIDAD *IN VITRO* Y EFECTO INMUNOMODULADOR DE PROTEÍNAS DE HUEVO PROCESADO

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Memoria presentada por:

Rodrigo Jiménez Saiz

Para optar al grado de

DOCTOR EN CIENCIA Y TECNOLOGÍA DE LOS ALIMENTOS

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Trabajo realizado bajo la dirección de:

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INFORMAN:

Que el presente trabajo titulado "Digestibilidad, alergenicidad *in vitro* y efecto inmunomodulador de proteínas de huevo procesado" y que constituye la Memoria que presenta Rodrigo Jiménez Saiz para optar al grado de Doctor, se ha realizado bajo su dirección en el Departamento de Bioactividad y Análisis de Alimentos del Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM).

Y para que conste firmamos el presente informe a 13 de febrero de 2012.

Fdo.: Elena Molina Hernández

Fdo.: Rosina López-Alonso Fandiño

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Amada Ciencia

En este absurdo mundo tú me amparas,
acertijo infinito, verdad prima,
alma máter, excelsa musa opima,
fanal de saber, prisma de mil caras.

Haces livianos los días, en aras
de un mundo mejor, con tu eterna rima,
apacible son de libros y enzimas,
más las tribulaciones que deparas.

Así se me va la vida, en ti habito,
con mi hábito que antes fue harto blanco
mas hoy se muestra gris, tan desgastado…

Sigo creyendo que el gris es bonito,
Y tú sigues sentada aquí en mi flanco,
¿habré vivido o tan solo ensuciado?

Rodrigo Jiménez Saiz

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$a_{w:}$ actividad de agua

DSC: calorimetría diferencial de barrido

ELISA: ensayo por inmunoabsorción ligado a enzimas

G: goma arábiga

IFN-γ: interferón gamma

Ig: inmunoglobulina

iT-regs: células T-reguladoras adaptativas

IL: interleucina

LYS: lisozima

ME: metil esterificación

nT-regs: célultas T-reguladoras naturales

OIT: inmunoterapia oral

OM: ovomucoide

OT: ovotransferrina

OVA: ovoalbúmina

O/W: emulsión de aceite en agua

P: pectina

PC: fosfatidilcolina

pI: punto isoeléctrico

RM: reacción de Maillard

RP-HPLC: cromatografía de alta eficacia en fase inversa

SDS-PAGE: electroforesis en gel de acrilamida con dodecil sulfato sódico

SEC: cromatografía de exclusión molecular

TGF-β: factor de crecimiento transformante beta

Th: linfocitos T de ayuda

Th-0: linfocitos T de ayuda vírgenes

Th-1: linfocitos T de ayuda de respuesta no alérgica

Th-2: linfocitos T de ayuda de respuesta alérgica

W/O: emulsión de agua en aceite

X: xilano

INTRODUCCIÓN

INTRODUCCION

1. Alergia a huevo

La relevancia del huevo, tanto en el ámbito nutricional como tecnológico, es indiscutible, dado que supone una excelente fuente de proteínas de alto valor biológico y presenta propiedades funcionales de gran utilidad para la industria alimentaria (Mine, 2002). La producción en España de 986 millones de docenas de huevos en el año 2008, según los datos ofrecidos por el Ministerio de Medio Ambiente y Medio Rural y Marino (MARM, 2009), refrenda su importancia. A pesar de tales ventajas, el huevo pertenece al grupo de "*los ocho*", que abarca aquellos alimentos mayormente implicados en alergias alimentarias incluyendo leche, cacahuete, frutos secos, trigo, pescado, marisco y soja (Bush y Hefle, 1996).

La alergia a huevo está clasificada como una reacción no tóxica de hipersensibilidad en la que está implicada el sistema inmune. Se trata de una respuesta inmune exacerbada y manifiesta frente a alérgenos de huevo—entendiéndose por antígenos las moléculas capaces de ser reconocidas por el sistema inmune y como alérgenos los antígenos capaces de desencadenar una respuesta alérgica—que de cualquier otro modo serían inocuos (Bischoff y Crowe, 2005; Holgate y Polosa, 2008). La alergia a huevo está mediada por anticuerpos denominados inmunoglobulina (Ig) E, lo que la ubica dentro de las reacciones de hipersensibilidad de tipo I, siendo esta la forma de alergia a alimentos más común y mejor caracterizada (Ebo y Stevens, 2001; Caubet y Wang, 2011).

1.1 Diagnóstico y prevalencia

La diagnosis de alergia a huevo es normalmente determinada por pruebas de reacción cutánea y ensayos de radioalergoabsorbencia—que miden niveles de IgE específicos en sangre—, sin embargo, la provocación oral doble ciego, controlada con placebo, es el patrón oro, ya que confirma el diagnóstico clínico. Dado que la provocación oral con huevo puede dar pie a reacciones adversas, se han establecido valores de corte para los niveles específicos de IgE determinados por ensayos de radioalergosorbencia, y para el diámetro de pápula medido en pruebas de reacción cutánea, pudiendo así identificar pacientes alérgicos a huevo sin necesidad de poner al afectado en una posible situación de riesgo (Heine y col., 2006). En los primeros estudios realizados con este fin, se concluyó que un nivel de IgE específica de huevo >6kU/L se correlacionaba con un valor predictivo

positivo >95% en niños y adolescentes (Sampson y Ho, 1997). En estudios clínicos más recientes, realizados en grupos de niños alemanes (Celik-Bilgili y col., 2005) y japoneses (Komata y col., 2007), se observó que estableciendo 12.6 y 25.5 kU/L respectivamente, como nivel de corte, se daba un 95% de valor predictivo positivo, mientras que otro estudio similar, efectuado en niños españoles menores de dos años, apuntó un nivel de corte bastante menor, situado en >0.36 kU/L, con un valor predictivo del 94% (Boyano Martínez y col., 2001). Respecto a pruebas de reacción cutánea, tampoco hay un consenso sobre qué diámetro de pápula fijar como valor predictivo positivo de alergia a huevo (Sporik y col., 2000; Verstege y col., 2005), aunque un diámetro de pápula ≥ 3mm se ha interpretado habitualmente como síntoma de alergia (Caubet y Wang, 2011).

La prevalencia estimada de alergia a huevo está comprendida entre 1.6 y 3.2% (Heine y col., 2006) lo que la sitúa, conforme a diversos estudios desarrollados en países de Europa (Gustafsson y col., 2003; Kristinsdottir y col., 2011), Asia (Chiang y col., 2007; Chen y col., 2011) y Oceanía (Osborne y col., 2011), como la mayor reacción de hipersensibilidad en la población pediátrica junto con la leche (en Europa y Asia), o con el cacahuete (en Oceanía). Las variaciones entre estudios son atribuidas a diferentes factores, como los parámetros elegidos para decidir si el individuo en cuestión es o no alérgico, las características clínicas de las cohortes estudiadas (edad, origen) y las características del alérgeno usado durante la provocación (naturaleza, procesado). Tales diferencias se ven reflejadas en la gran heterogeneidad de resultados publicados sobre la prevalencia de alergia a huevo (Rona y col., 2007). Además, la ingesta de huevo puede causar anafilaxis en pacientes altamente sensibilizados (Colver y col., 2005; Ross y col., 2008), induciendo reacciones severas que pueden llegar a ocasionar la muerte (Mehl y col., 2005).

1.2 Mecanismos inmunológicos

1.2.1 Mucosa intestinal: transporte y sensibilización

Las reacciones de hipersensibilidad a alimentos mediadas por IgE pueden ocurrir después de la exposición al antígeno por diferentes rutas entre las que destacan la vía gastrointestinal, la vía respiratoria y la vía cutánea (Chapman y col., 2006); estando la presente tesis doctoral enmarcada en la sensibilización a huevo a través del sistema digestivo. Durante muchos años se presumió que las macromoléculas no eran capaces de llegar al torrente sanguíneo por el tracto gastrointestinal, apoyándose en la idea de que la presencia de secreciones mucosas, IgA y bacterias comensales se comportaban en el intestino a modo de barrera. Aun así, en la actualidad es sabido que los antígenos dietarios

pueden pasar a través del epitelio intestinal tanto en su forma nativa como parcialmente digeridos y, por tanto, interaccionar directamente con las células del sistema inmune (Chehade y Mayer, 2005). También se sabe que la absorción de antígenos alimentarios es más significativa en niños que en adultos, con un descenso de las titulaciones de anticuerpos específicos de alimentos a partir del primer año de vida (Burks y Sampson, 1993), lo que puede ser debido a la maduración del epitelio intestinal (Vukavić, 1984).

Los antígenos alimentarios pueden atravesar el intestino y entrar en contacto con el sistema inmune mediante múltiples mecanismos, que se hallan representados en la **Figura 1**, entre los cuales está el transporte del antígeno alimentario a través de las uniones celulares del epitelio intestinal (Fig. 1, A); la invaginación, proceso más conocido por endocitosis (Fig.1, B); la absorción mediada por células especializadas, llamadas células M, las cuales están estratégicamente localizadas sobre las placas de Peyer y permiten la liberación del antígeno directamente en células linfáticas (Fig. 1, C); alternativamente existe la posibilidad de que las células dendríticas se extiendan a través del epitelio alcanzando el lumen y tomen directamente los antígenos alimentarios (Fig. 1, D) (Chehade y Mayer, 2005).

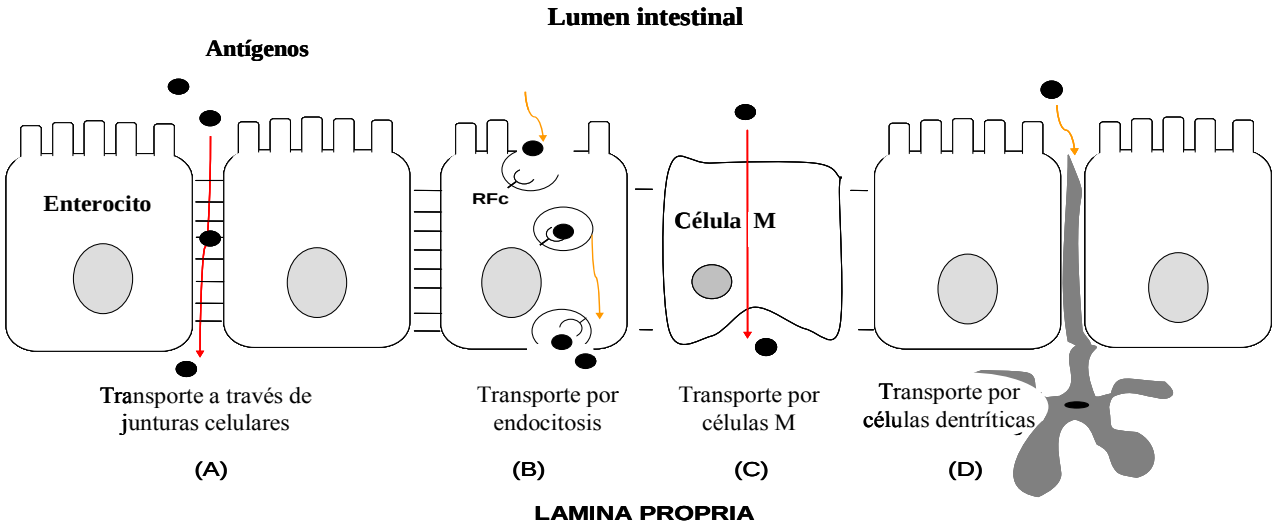


Figura 1. Diferentes rutas de transporte de antígenos en la barrera intestinal.

El paso de antígenos a través de la barrera intestinal puede ser la génesis de la hipersensibilidad a alimentos, que es comúnmente descrita como un fenómeno que consta de dos fases. En la primera fase, denominada fase de sensibilización, los antígenos alimentarios traspasan la barrera intestinal y son capturados por las células inmunes subyacentes. Estos son subsecuentemente procesados y llevados a células inmunes

especializadas, conocidas como células presentadoras de antígenos, para finalmente entrar en contacto con linfocitos T de ayuda (Th). Las citoquinas son proteínas que regulan las funciones celulares, por tanto, el perfil de citoquinas presente en el medio en el que se hallan los linfocitos T vírgenes (Th-0) juega un papel prominente para determinar si estos expresarán un fenotipo Th-1 o un fenotipo Th-2. Los individuos alérgicos desarrollan preferentemente un entorno rico en interleucina (IL) 4, que es la citoquina distintiva de la respuesta alérgica (Th-2) y estimula la diferenciación de linfocitos Th-0 en Th-2, así como la producción de anticuerpos IgE e IgG_1. Otras citoquinas partícipes de la respuesta Th-2 son la IL-13, que también promueve la producción de IgE, la IL-5, que estimula la proliferación de eosinófilos, y la IL-9, que participa en la activación de mastocitos. Además, la IL-25, IL-31 y IL-33 se han asociado recientemente con la respuesta Th-2 (Akdis y Akdis, 2011). El aumento de la respuesta inmune Th-2 va en detrimento de la respuesta Th-1, que predomina habitualmente en individuos no alérgicos. El IFN-γ es la citoquina más representativa de la respuesta Th-1, y destaca por promover la diferenciación de células Th-0 en Th-1 e impedir la de células Th-2, además de inhibir la producción de IgE e IgG_1 y estimular la producción de otras subclases de IgG. El IFN-γ también fomenta la producción de IL-12, que promueve la diferenciación de células Th-0 en Th-1 y a su vez aumenta a la secreción de IFN-γ (Chehade y Mayer, 2005; Abbas y col., 2009).

El desvío hacia una respuesta Th-2 estimula la producción en linfocitos B de IgE específicas del alérgeno; estas se unen a los receptores de alta afinidad (FcεRI) presentes en la superficie de los mastocitos y basófilos, y cuando se repite la ingestión del alérgeno ocurre la segunda fase de la respuesta alérgica, conocida como fase efectora. Los fragmentos alergénicos se unen a las IgE presentes en mastocitos y basófilos, desencadenando la agregación de los receptores y la liberación de mediadores inflamatorios y vasoactivos, fundamentalmente leucotrienos, prostaglandinas e histamina. Posteriormente, la presentación del antígeno conllevará una rápida activación de células Th-2, así como el reclutamiento y la activación de células efectoras como eosinófilos y basófilos (Abbas y col., 2009).

1.2.2 El balance Th-1/Th-2 y las células T-reguladoras

Durante un largo periodo de tiempo, la opinión dominante fue que el balance Th-1/Th-2 jugaba un papel central en la regulación de las respuestas inmunes (Romagnani, 1991). Sin embargo, el modelo no era capaz de explicar aquellos casos en los que se producía la inhibición de ambas respuestas y que podía asociarse con un estado de

tolerancia. Ulteriormente, tal situación fue explicada por la actividad del heterogéneo grupo de células T reguladoras (T-regs). Las células T-regs proceden fundamentalmente de dos linajes, aquellas que derivan naturalmente del timo (nT-regs), y las que son inducidas o adaptativas (iT-regs), pues adquieren funcionalidad después de la estimulación antigénica y en presencia de un perfil de citoquinas concreto (Rolland y col., 2010). Estas últimas desempeñan un papel determinante en la respuesta inmune frente antígenos exógenos como son los de la dieta (**Figura 2**).

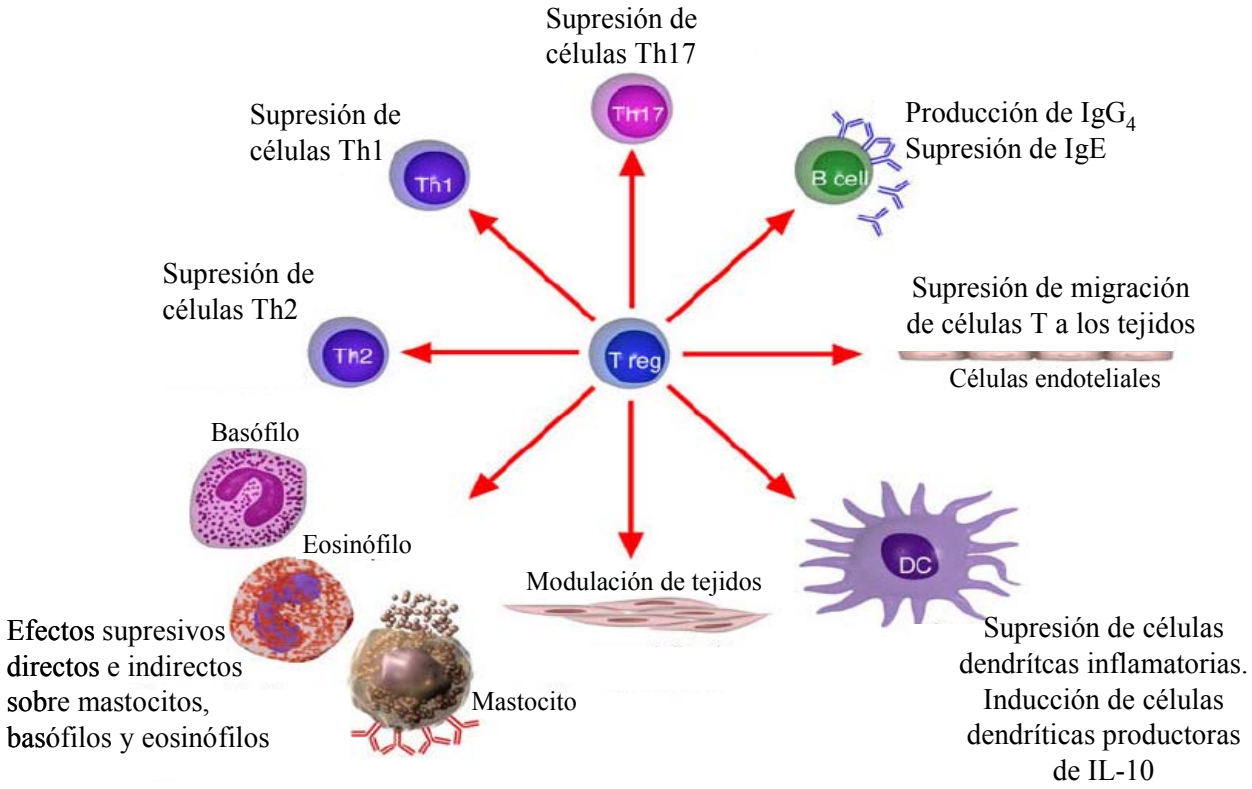


Figura 2. Mecanismos de células T-regs que contribuyen al control de respuestas inmunes frente a antígenos exógenos. Adaptada de Akdis y Akdis (2011).

Las células T-regs han demostrado ser capaces de modular la respuesta alérgica al inhibir células Th-1 y Th-2, ya sea por contacto directo o por producción de citoquinas supresivas como IL-10 y TGF-β (Stock y col., 2006). La IL-10 ha sido descrita como esencial en la tolerancia periférica a alérgenos y se le atribuyen, entre otras funciones, la de suprimir la síntesis de IgE, reducir la liberación de citoquinas proinflamatorias en los mastocitos, inducir la producción de IL-10 en células dendríticas y estimular la producción de IgG_4 y IgA (O'Garra y col., 2008). El TGF-β también puede contribuir a la tolerancia periférica de alérgenos al actuar sinérgicamente con la IL-10, reducir la síntesis de IgE,

suprimir la funcionalidad de células Th-1, Th-2 y Th-17, participar en la diferenciación de células Th-2 en Th-9 y Th-17 e inducir la síntesis de IgA (Letterio y Roberts, 1998). El descubrimiento de las células Th-9 y Th-17 es relativamente reciente, estando ambas implicadas en procesos inflamatorios (Dardalhon y col., 2008; Veldhoen y col., 2008).

Es interesante ver, entre las funciones atribuidas a IL-10 y TGF-β, la de producir IgA, pues se ha barajado durante mucho tiempo la posibilidad de que el desarrollo de una respuesta alérgica pueda estar relacionada con los niveles intestinales de IgA. De hecho, en modelos animales de alergias alimentarias, se han asociado bajas concentraciones de IgA en el intestino con el desarrollo de alergia, sugiriendo el papel fundamental de dicha inmunoglobulina en el desarrollo de la tolerancia periférica. Las placas de Peyer están directamente implicadas en la síntesis de IgA, estimuladas por la presencia de IL-10 y TGF-β, por tanto, la pérdida de la actividad de las células T-regs podría conllevar un aumento en el peso de la respuesta alérgica Th-2; dado que tanto individuos sanos como alérgicos muestran los tres repertorios de células T específicas del alérgeno en cuestión —repertorio Th-1, Th-2 y T-reg—es factible que las variaciones en la proporción de las mismas determinen si se desarrolla una respuesta inmune sana o inicua, marcada vehementemente por el ratio de células especificas iT-reg y Th-2 (Akdis y col., 2005).

1.3 Alérgenos de huevo

En general, los alérgenos alimentarios son proteínas de la dieta con una masa molecular comprendida entre 10 y 70 kDa (Mills y col., 2004), aunque hay excepciones, como el alérgeno Ara h 1 del cacahuete, con una masa molecular mayor de 250 kDa (Maleki y col., 2000). Las proteínas causantes de reacciones alérgicas suelen ser abundantes en el alimento, por ejemplo, los alérgenos mayoritarios de la leche, huevo, pescado y cacahuetes comprenden del 25 al 50% del total de proteínas presentes en alimento. También han de poseer cierta estabilidad estructural ya que, para sensibilizar a un individuo vía gastrointestinal, deben ser capaces de resistir el procesado y preservar en parte sus epítopos—que son aquellas regiones de la proteína implicadas en la respuesta alérgica—durante la digestión, soportando pHs ácidos, proteolisis, surfactantes, etc (Astwood y col., 1996); de hecho, muchos de los alérgenos alimentarios son proteínas estables, resistentes al calentamiento, como la β-lactoglobulina de la leche (Wal, 1998), y a la acción de enzimas digestivas, como la parvalbúmina de bacalao (Bugajska-Schretter y col., 1998). Además, se sabe que muchas de las proteínas alergénicas tienen punto

isoeléctrico (pI) ácido y contienen modificaciones translacionales, como fosforilación o adición de fracciones glicosiladas en su estructura (Bredehorst, 2001).

El huevo presenta alérgenos tanto en la clara como en la yema aunque se sabe que el potencial alergénico del huevo se halla fundamentalmente en la clara. Ya en los años cincuenta se trataba de identificar alérgenos de la clara de huevo realizando test de reacción cutánea a pacientes alérgicos a huevo (Miller y Campbell, 1950). En los años ochenta se emplearon técnicas de radioalergosorbencia y radioinmunoelectroforesis cruzada, estableciéndose la ovoalbúmina (OVA o Gal d 2), el ovomucoide (OM o Gal d 1) y la ovotransferrina (OT o Gal d 3) como alérgenos mayoritarios (Hoffman, 1983). Posteriormente, la lisozima (LYS o Gal d 4) fue también reconocida como alérgeno mayoritario del huevo (Holen y Elsayed, 1990). En un esfuerzo por establecer cuales de los alérgenos de huevo eran verdaderamente alérgenos mayoritarios, se llevó a cabo un estudio centrado en evaluar la unión específica de IgE frente a 8 proteínas de huevo usando suero de 48 niños alérgicos (Walsh y col., 2005). Tal estudio confirmó que los alérgenos mayoritarios de huevo se hallaban fundamentalmente en la clara de huevo e incluían OVA, OM, OT y LYS. En la actualidad es ampliamente aceptado que la OVA y el OM son los alérgenos principales del huevo (Mine y Yang, 2008) debido a sus propiedades moleculares e inmunológicas, así como a su comportamiento frente al procesado, aspectos que son revisados en los siguientes apartados.

1.3.1 Ovoalbúmina

La OVA es una fosfoglicoproteína de una masa molecular de 45 kDa y es la proteína más abundante de la clara de huevo, contando con un 54% (p/p) del contenido proteico. La secuencia completa de 385 aminoácidos ha sido descrita (McReynolds y col., 1978; Nisbet y col., 1981) y su estructura tridimensional ha sido estudiada por cristalografía de rayos X, observándose tres láminas β y nueve hélices α (Stein y col., 1990). La OVA contiene una unidad de carbohidratos, puede presentar hasta dos residuos de fosfoserina, un puente disulfuro y cuatro grupos sulfidrilo, tres de los cuales son poco reactivos en la forma nativa, mientras que el cuarto es altamente reactivo cuando la proteína se desnaturaliza. La mitad de los residuos de aminoácidos de la OVA son hidrofóbicos y un tercio presenta residuos cargados, la mayoría ácidos, confiriéndole a la proteína un pI de 4.5 y una temperatura de desnaturalización de 84ºC (Li-Chan y Nakai, 1989).

Un hecho interesante en la estructura de la OVA es su homología con la superfamilia de las serpinas (serinas inhibidoras de proteasas), que son un grupo de proteínas, presente en todos los organismos eucariotas, con actividad inhibidora de proteasas (Huntington y Stein, 2001). No obstante, la OVA no muestra tal actividad inhibidora de proteasas y, aparte su papel como fuente importante de aminoácidos, no se le han atribuido funciones biológicas todavía. La OVA ha sido ampliamente usada, no solo como modelo estándar para estudios estructurales y funcionales de proteínas, sino como antígeno modelo en modelos animales experimentales de alergias alimentarias y asma, habiendo sido identificados los epítopos dominantes de unión a IgE en humanos y en ratones (Mine y Rupa, 2003; Mine y Yang, 2007).

1.3.2 Ovomucoide

El OM tiene una masa molecular de 28 kDa y un pI de 4.82. Representa el 11% (p/p) de las proteínas de la clara de huevo. Se encuentra altamente glicosilado, conteniendo un 20-25% de fracciones de carbohidratos. El OM comprende 186 aminoácidos organizados en tres dominios estructuralmente independientes (Gal d 1.1, 1.2 y 1.3), y posee 9 puentes disulfuro intermoleculares. El OM presenta capacidad de reducir la actividad de algunas enzimas digestivas, como la tripsina y quimotripsina (Konishi y col., 1985), así como un gran potencial alergénico. El dominio Gal d 1.3 es considerado como la fracción inmunodominante del OM en humanos alérgicos a huevo ya que mostró mayor actividad de unión a IgE e IgG que los dominios I y II (Zhang y Mine, 1998). Las propiedades alergénicas del OM se han descrito extensamente y los epítopos de células B y T han sido identificados tanto en pacientes alérgicos a huevo como en ratones (Holen y col., 2001; Mine y Zhang, 2002; Mizumachi y Kurisaki, 2003).

1.3.3 Lisozima

La LYS, aun no siendo tan alergénica como la OVA o el OM, es ampliamente usada por sus propiedades antibacterianas para prevenir infecciones, lo que aumenta el riesgo de exposición y hace que sus propiedades alergénicas despierten mayor interés. Las LYS es una proteína de 129 aminoácidos y masa molecular de 14.3 kDa (Mine y Yang, 2008). Es de carácter básico, con un pI de 10.7; presenta 4 puentes disulfuro en su estructura que confieren estabilidad y, a diferencia de OVA y OM, no tiene carbohidratos en su estructura. Fue la primera proteína en ser secuenciada y su estructura tridimensional fue analizada viéndose que consiste en dos dominios unidos por una α-hélice, dónde se

encuentra el sitio activo de la enzima (Young y col., 1994). Sus grupos polares se encuentran en la superficie, mientras que la mayoría de los hidrófobos están en el interior. Representa un 3.5% del contenido proteico de la clara de huevo, y se caracteriza por su capacidad antibacteriana, especialmente frente a bacterias gram positivas, dada su habilidad para hidrolizar el enlace glucosídico. También es señalada por su potencial alergénico, habiéndose realizado algunos estudios en ratones para identificar epítopos de células T (Gammon y col., 1991; Moudgil y col., 1997), pero no en humanos, estando la caracterización de epítopos de LYS relevantes en la alergia a huevo incompleta.

2. Efecto del procesado en alérgenos alimentarios

Los alimentos e ingredientes alimentarios están sujetos a una gran variedad de condiciones de procesado enfocadas a mejorar las propiedades organolépticas y/o funcionales, asegurar la calidad microbiológica del alimento, modificar sus características para adecuarlos a su uso final o aislar determinados compuestos del alimento inicial (Thomas y col., 2007). El procesado de alimentos puede inducir cambios físicos, químicos y físico-químicos que afecten sensiblemente el potencial alergénico de las proteínas alimentarias mediante el efecto provocado en su estructura dependiendo del tipo de procesado, duración e intensidad (Wal, 2003; Sathe y col., 2005).

Generalmente, a través del procesado de alimentos se puede disminuir la alergenicidad, ya sea por destrucción, modificación o enmascaramiento de epítopos. Se habla de epítopos conformacionales (**Figura 3**) cuando se hallan formados por diversas regiones separadas en la secuencia proteica primaria, que se aproximan por el plegamiento tridimensional de la proteína; por tanto, los cambios conformacionales causados por el procesado suelen ser críticos para esta clase de epítopos, aunque no se puede descartar una posible formación de nuevos puntos de

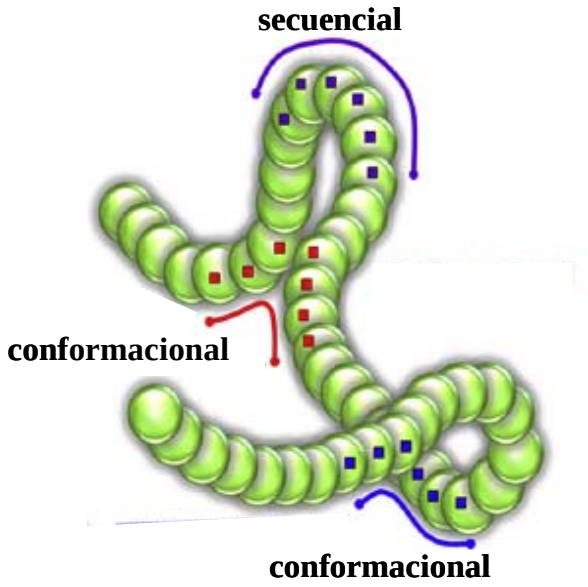


Figura 3. Clases de epítopos. Adaptado de Konstantinuo y col. (2012).

unión a IgE dependiendo del desplegamiento de la proteína y de su reorganización espacial. Se habla de epítopos lineales **(Figura 3)** cuando se encuentran formados por aminoácidos contiguos en la secuencia primaria de la proteína. Los epítopos lineales son críticos en aquellos casos de alergia persistente y es más factible que estos se alteren, por ejemplo, por hidrólisis enzimática que por cambios conformacionales (Urisu y col., 1999).

Otro aspecto fundamental en la compresión del efecto del procesado sobre el potencial alergénico de un alimento es cómo afecta el procesado la digestión gastrointestinal del alérgeno, ya que puede modificar la susceptibilidad a la hidrólisis del mismo. Por ejemplo, en el caso de la β-lactoglobulina, presente en el suero lácteo, el tratamiento térmico no es suficiente para eliminar su alergenicidad, mas consigue aumentar su digestibilidad, reduciendo notablemente su capacidad de desencadenar una respuesta alérgica (Ehn y col., 2004). La resistencia a la digestión gastrointestinal se considera una característica importante de los alérgenos alimentarios, que deben ser capaces de conservar los epítopos, a pesar de las condiciones ácidas y enzimáticas del aparato digestivo, y alcanzar el sistema inmune a través del intestino con capacidad de desencadenar un respuesta alérgica (Astwood y col., 1996).

2.1 Tratamiento térmico

De modo general, los tratamientos térmicos son aplicados a los alimentos con dos objetivos: mantener o mejorar la calidad microbiológica del alimento (pasteurización, esterilización, refrigeración, congelación) y/o conseguir la adecuación culinaria para su consumo (asado, hervido, cocido, frito, escalfado, ahumado, secado, aliñado, etc.). El tratamiento térmico de las proteínas alimentarias produce diversas modificaciones abarcando desnaturalización, hidrólisis de enlaces peptídicos, agregación por enlaces no covalentes y puentes disulfuro, y reacciones con otras moléculas del alimento ya sean lípidos o carbohidratos. Dependiendo de la proteína, se sabe que la pérdida de la estructura secundaria ocurre a 55-70ºC, la rotura de puntes disulfuro a 70-80ºC, la formación de nuevas interacciones intra o inter moleculares y reordenamiento de los puentes disulfuro a 80-90ºC y agregación a 90-100º con la subsiguiente pérdida de la estructura terciaria (Wal, 2003). Como cabe esperar, este amplio abanico de reacciones puede afectar ostensiblemente la alergenicidad de las proteínas. Por ejemplo, los alérgenos Mal d 1 de la manzana (Scheurer y col., 2004), Pru av 1 de la cereza (Bohle y col., 2006), Api g 1 del apio (Jankiewicz y col., 1997) y Cor a 1.04 de avellana (Pastorello y col., 2002) ven

reducida su alergenicidad por la pérdida de epítopos conformacionales debido al desdoblamiento de la proteína calentada. En contraposición, también puede producirse el efecto opuesto y que se incremente la alergenicidad por favorecer el acceso a epítopos encriptados tras desdoblarse la proteína, o por formación de nuevos epítopos a consecuencia de plegamientos tras la desnaturalización del alérgeno (Besler y col., 2001; Molina y col., 2009). Además, no se puede obviar el efecto del procesado sobre la digestibilidad, como mencionábamos con anterioridad.

En el huevo, el tratamiento térmico se suele utilizar para mejorar la textura, el sabor, o por seguridad microbiológica, y no con la finalidad de reducir su alergenicidad. No obstante, el efecto del tratamiento térmico en el huevo ha sido ampliamente estudiado; por ejemplo, al comienzo de los año de los ochenta, se publicó que el huevo hervido (100ºC durante 3 y 20 min) mostraba una antigenicidad reducida (Hoffman, 1983). En estudios *in vitro* con clara de huevo se aportaron resultados parecidos: al calentar la clara a 90ºC durante 10 min la capacidad de unión a IgE de 16 pacientes alérgicos a huevo disminuyó por encima del 50% (Anet, 1985). Más recientemente, un estudio realizado en dos pacientes que habían sufrido reacciones anafilácticas al ingerir huevo crudo reveló que tales afecciones no se reproducían cuando lo consumían cocinado (Eigenmann, 2000). En la misma línea, se han realizado estudios clínicos en los que concluyeron que alrededor de un 70% de los niños alérgicos a huevo eran capaces de tolerar huevo como ingrediente de distintos tipos de bollería (Roches y col., 2006; Lemon-Mule y col., 2008). En un estudio actual, y de acuerdo con lo descrito en la bibliografía, se observó que un 66% de niños alérgicos a huevo ingerían clara de huevo pasteurizada sin presentar síntomas de alergia (Jurado-Palomo y col., 2010).

Si centramos la revisión bibliográfica en los alérgenos principales del huevo, OVA y OM, encontramos que sus comportamientos difieren cuando son sometidos a tratamiento térmico. La OVA pierde una notable capacidad de unión a IgE por calentamiento a 80ºC durante 3 minutos (Honma y col., 1994), pero el hecho de que presente en su estructura tanto epítopos lineales como conformacionales, hace posible que mantenga cierta alergenicidad después del tratamiento térmico (Hoffman, 1983; Mine y Zhang, 2002). Otro aspecto importante, como anotábamos previamente, es la estabilidad del alérgeno frente al proceso digestivo. En un primer momento, fue publicado que la OVA era estable a la acción de la tripsina, pero susceptible a la de la pepsina (Elsayed y col., 1986), aunque finalmente se ha comprobado que es muy estable en medio gástrico y duodenal (Martos y col., 2010). En cambio, cuando es sometida a tratamiento térmico (100ºC durante 5 min), la

digestibilidad aumenta notablemente, tanto a nivel gástrico como duodenal (Takagi y col., 2003), lo cual podría reducir su potencial alergénico.

A diferencia de la OVA, el OM no es coagulable por calor (Urisu y col., 1997) y retiene alergenicidad después de calentamiento prolongado a elevada temperatura (Gu y col., 1986). Tal estabilidad es atribuida al alto contenido de carbohidratos y los nueve puentes disulfuro presentes en su estructura (Hirose y col., 2004). Urisu y col. (1997) dirigieron un estudio clínico en el que se administró clara de huevo calentada (90ºC durante 60 min) con el contenido de OM habitual, o habiéndose reducido, a pacientes alérgicos a huevo. La mitad de los pacientes toleraron la clara de huevo calentada mientras que la clara de huevo calentada con bajo contenido en OM fue tolerada por un 95% de los pacientes. Este estudio reveló la importancia del OM en la alergia a huevo, posiblemente debido a su estabilidad térmica, en comparación con otros alérgenos reconocidos de la clara de huevo que son termolábiles, como la OVA, OT y LYS. Respecto a la estabilidad del OM en medio gastrointestinal, se sabe que se digiere con pepsina dando lugar a tres bandas observadas por SDS-PAGE, mas tales bandas muestran estabilidad en medio duodenal, lo cual puede ser en parte atribuible a su actividad inhibidora de tripsina y quimotripisina (Kovacs-Nolan y col., 2000). La gran estabilidad que exhibe el OM frente al tratamiento térmico hace pensar que este no modificará su digestibilidad, sin embargo, el calentamiento puede favorecer la interacción del mismo con otro tipo de moléculas presentes en la matriz del huevo, como carbohidratos y/o lípidos, produciendo modificaciones estructurales que podrían afectar finalmente su alergenicidad y digestibilidad.

2.2 Almacenamiento

El almacenamiento de los alimentos puede dar lugar a modificaciones estructurales de los alérgenos que contienen hasta que son consumidos. En el caso del huevo, es sabido que la OVA sufre cambios estructurales durante el almacenamiento, generando la llamada S-OVA (Smith y Back, 1965). La formación de S-OVA conlleva una serie de cambios conformacionales, que todavía son objeto de investigación, aunque se acepta que la estructura de la OVA y la S-OVA son muy semejantes. No obstante, se han descrito diferencias: la Ser 164, Ser 236, Ser 320 toman la configuración D y las cadenas laterales de Phe 99 y Met 241 sufren alteraciones significativas en la S-OVA (Yamasaki y col., 2003; Ishimaru y col., 2010); también hay un incremento de láminas β antiparalelas junto con un descenso de hélices α (Kint y Tomimatsu, 1979). Dichas modificaciones resultan en una mayor termoestabilidad de la S-OVA, que presenta una

temperatura de desnaturalización de 92.5ºC, determinada por calorimetría diferencial de barrido (DSC) (Donovan y col., 1975). La formación de S-OVA es progresiva, pudiendo distinguirse formas intermedias entre OVA y S-OVA hasta que la formación completa de esta última se consuma. Los estados intermedios pueden ser fácilmente distinguidos por DSC (Hegg y col., 1979).

En la clara de huevo fresca puede haber hasta un 5% de S-OVA, aunque más de la mitad de la OVA se convierte en S-OVA en el tiempo transcurrido hasta que los huevos llegan al consumidor (Lechevalier y col., 2007). Después de seis meses de almacenamiento a baja temperatura el porcentaje de S-OVA puede alcanzar un 81% (Vadehra y Nath, 1973). La S-OVA también se puede formar *in vitro* en medio alcalino y elevada temperatura. Por ejemplo, Donovan y col. (1975) prepararon S-OVA rápidamente, incubando OVA a 50ºC durante 20 horas en tampón fosfato sódico 100 mM y pH 10.

La formación de S-OVA está normalmente asociada con una pérdida de funcionalidad de la clara de huevo, ya que reduce la consistencia de la clara y su capacidad coaguladora (Shitamori y col., 1984). La mayoría de los estudios llevados a cabo sobre la S-OVA se han centrado en la calidad organoléptica de los huevos almacenados y de los productos relacionados (Huntington y Stein, 2001), mas no en aspectos relacionados con el potencial alergénico de la S-OVA ni en su estabilidad en medio gastrointestinal, siendo estos de interés, dado que la S-OVA se ha descrito como una forma más estable que la OVA y dicha estabilidad podría también manifestarse a nivel intestinal resultando en una mayor alergenicidad.

3. Efecto de la matriz

El comportamiento de los alérgenos en la matriz alimentaria se ha convertido recientemente en un candente tema de investigación. Usualmente se afronta el estudio de alérgenos alimentarios a partir de extractos o formas puras, lo que facilita la tarea. Sin embargo, raros son los casos en los que nos exponemos al alérgeno en una forma purificada o aislada. Por tanto, el alcance de conclusiones más relevantes, en cuanto a la alergenicidad de las proteínas alimentarias y su comportamiento en condiciones fisiológicas, requiere el estudio de las mismas en las matrices en las que son habitualmente procesadas y consumidas, o al menos en modelos experimentales que asemejen tales condiciones. Se sabe que los alérgenos alimentarios pueden interaccionar con otros compuestos que estén en la matriz, siendo los más habituales carbohidratos y

lípidos. Tales interacciones pueden resultar en un aumento o descenso del potencial alergénico y afectar el modo en el que son digeridos y/o reconocidos los alérgenos a nivel celular y, por ende, su capacidad de desencadenar una respuesta inmune (Thomas y col., 2007) (**Figura 4**).

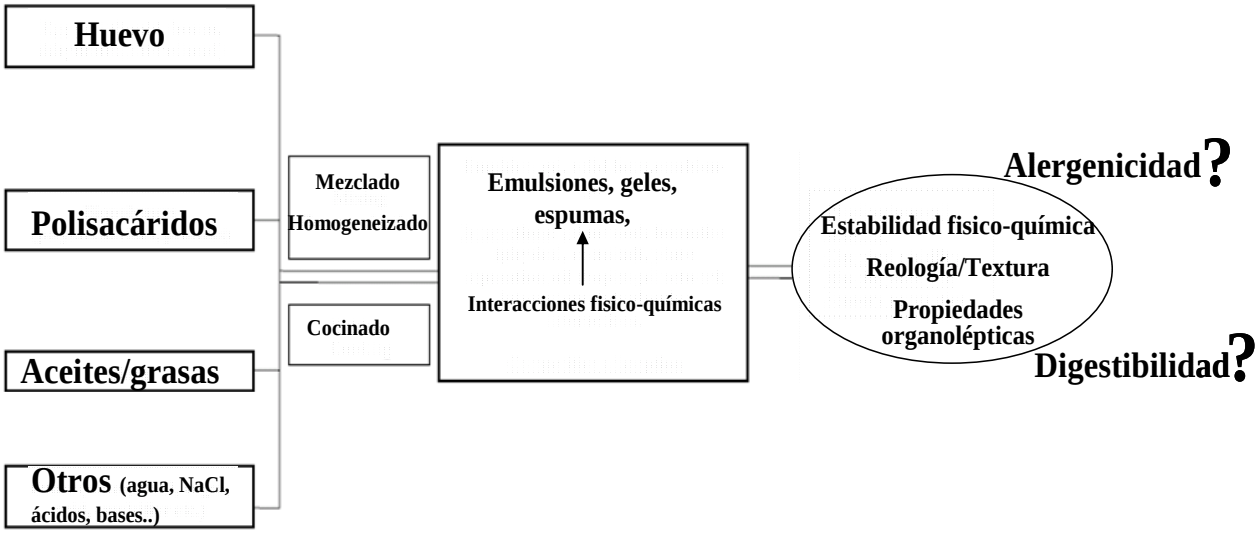


Figura 4. Interrogantes relativos a la matriz y procesado del huevo.

3.1 Interacción con carbohidratos

3.1.1 Reacción de Maillard (RM)

La RM (Maillard, 1912) es una reacción no enzimática, que sucede entre grupos amino libres y azúcares reductores en los alimentos durante el tratamiento térmico o almacenamientos largos, bajo unas condiciones determinadas (Nursten, 1981). Es un denso entramado de reacciones que ha sido objeto de numerosos estudios (Friedman, 1996; Martins, 2000; Jiménez-Castaño y col., 2007) y consta de tres etapas: inicial, intermedia y final (Hodge, 1953).

La etapa inicial de la RM es la mejor caracterizada, comienza con la condensación del grupo carbonilo de un azúcar reductor con un grupo amino libre, liberándose agua para formar una base de Shiff, que sufre una posterior ciclación transformándose en una glicosilamina N-sustituida inestable, que finalmente se reordena irreversiblemente dando el compuesto de Amadori, cuando el azúcar reaccionante es una aldosa, o el compuesto de Heyns, si el azúcar reaccionante es una cetosa (Matsuda y col., 1991). Durante la primera etapa las proteínas no sufren alteraciones estructurales importantes ni se produce formación de color (Oliver,

2006). Durante la etapa intermedia se produce la fragmentación de azúcares y la degradación de aminoácidos (degradación de Strecker). El compuesto de Amadori o Heyns, puede experimentar varias reacciones de degradación irreversibles, generándose una gran variedad de compuestos que todavía están pobremente caracterizados (Ledl, 1990). La etapa final de la RM supone una serie de reacciones de ciclación, deshidratación, retroaldolización, reordenamiento, isomerización y condensación de los productos iniciales de la reacción, dando lugar a la formación de compuestos poliméricos, coloreados, e insolubles en agua, denominados melanoidinas (Friedman, 1996).

Los factores principales que afectan el desarrollo de la RM han sido estudiados, siendo temperatura y tiempo de calentamiento, a_w, pH del medio, y naturaleza y concentración de los reactantes los más importantes. La temperatura y tiempo de calentamiento son considerados los factores más determinantes de la RM, estando ampliamente aceptado que el incremento de la temperatura, así como el tiempo de almacenamiento, favorecen de forma exponencial la RM (Ryu y col., 2003).

En general, el aumento de la a_w produce un incremento de la RM, hasta alcanzar un máximo, a partir del cual disminuye la reacción, posiblemente debido a la dilución de los reactivos. Diversos autores han demostrado que el intervalo óptimo de a_w es entre 0.3 y 0.7 (Labuza, 1977). El desarrollo de la RM está fuertemente condicionado por el pH, así como por la capacidad tamponadora del sistema. En condiciones ácidas (pH 3) la velocidad de la reacción es mínima y su desarrollo escaso (Lea y Hannan, 1949). Por lo general, la velocidad de la reacción aumenta en condiciones ligeramente alcalinas (Namiki y col., 1993; Ajandouz y Puigserver, 1999) considerándose el rango de pH 6 a 8 es idóneo para el desarrollo de la RM, pudiéndose ampliar hasta pH 10.

Por supuesto, la naturaleza y concentración de los reactantes influye en la RM. Los grupos de aminoácidos más reactivos son el ε-amino libre de Lys y α-amino terminal. Por ende, con un mayor contenido en residuos de Lys podría alcanzarse un mayor grado de glicación de la proteína. El grupo imidazol de la His, el grupo indol del Trp, y el grupo guanidino de los residuos de Arg también son susceptibles de reaccionar con azúcares reductores, pero en menor medida (Oliver, 2006). Se sabe que la OVA contiene 20 Lys y 15 Arg mientras que el OM presenta,

respectivamente, 14 y 5 (www.rcsb.org). Estos aminoácidos tienen grupos amino libres que podrían participar en la RM, aunque con distinta disponibilidad.

Respecto al carbohidrato, se ha descrito que la reactividad de los azúcares reductores disminuye al aumentar el peso molecular (Nacka y col., 1998). A su vez, la reactividad de los monosacáridos va a afectar la RM, siendo las aldosas intrínsecamente más reactivas que las cetosas (Yeboah y col., 1999), aunque se ha descrito que las cetosas podrían dan lugar a un mayor entrecruzamiento y agregación proteica durante las etapas avanzadas de la RM (Sun y col., 2004; Sun y col., 2006). Obviamente, la concentración de reactantes afecta la reacción, viéndose favorecida con exceso de azúcares reductores en el medio (Warmbier y col., 1976).

3.1.2 Influencia de la reacción de Maillard en la alergenicidad

La RM es una de las reacciones químicas principales que pueden influir en la alergenicidad de las proteínas alimentarias. La unión de polisacáridos a proteínas por RM va a ser más limitada que la de oligosacáridos, fundamentalmente por impedimento estérico (Jiménez-Castaño y col., 2005). Se sabe que la unión de hidratos de carbono a proteínas alimentarias puede variar su alergenicidad ya sea modificando la estructura terciaria con la subsiguiente destrucción o enmascaramiento de epítopos conformacionales, creando nuevos sitios de unión a IgE o exponiendo aquellos que estuviesen ocultos en la estructura. Se ha descrito que la RM puede generar nuevos sitios de unión a IgE en los alérgenos del cacahuete (Maleki y col., 2000; Gruber y col., 2005). En cambio, también se ha descrito que puede disminuir la capacidad de unión a IgE, como es el caso del alérgeno mayoritario de la cereza, Pru av 1, que ve reducida tal capacidad cuando es glicosilado con glucosa y ribosa (Gruber y col., 2004). Además se ha barajado la posibilidad de que los nuevos antígenos formados por glicación se unan a receptores diferentes, con lo cual, su reconocimiento, transporte y presentación por células presentadoras de antígenos seguiría otras vías de señalización distintas, resultando en respuestas inmunes diferentes respecto de la proteína nativa (Maleki y col., 2000).

Otro aspecto a tener en cuenta es que los cambios conformacionales y físico-químicos relacionados con la glicación pueden modificar la susceptibilidad de la proteína a la acción de las enzimas digestivas, así como su absorción intestinal, con el subsiguiente efecto en el potencial alergénico (Corzo-Martinez y col., 2010). Muchas proteínas modificadas mediante RM han mostrado mayor resistencia al proceso

digestivo, probablemente porque la reactividad de tripsina es menor frente a residuos de Lys y Arg glicados en comparación con los nativos (Moreno y col., 2008). En contraposición, ha sido publicado que los estados avanzados de la RM pueden favorecer la digestibilidad de las proteínas al exponer partes de la proteína susceptibles de ser hidrolizadas, debido a los cambios conformacionales producidos durante la glicosilación (Yeboah y col., 2004).

Las proteínas de la clara de huevo se desnaturalizan y vuelven insolubles a temperaturas relativamente bajas, por ejemplo, a pH neutro la OT se desnaturaliza a 61ºC y la LYS a 75ºC (Donovan y col., 1975); por esta razón, a nivel industrial es preferido el proceso de atomización. Durante este proceso es factible que la glucosa presente en la clara, alrededor de un 4% del peso en sólidos, reaccione con los grupos amino libres de los alérgenos principales, OVA y el OM, pudiendo desarrollarse colores y sabores no deseados, incluso después de periodos cortos de almacenamiento a temperatura ambiente. Por ello, en la práctica industrial, la clara de huevo es sometida a un proceso de eliminación del poder reductor antes de ser atomizada, para proteger el producto de la RM durante el calentamiento y el almacenado (Sisak y col., 2006). No obstante, durante el proceso de secado y ulterior almacenamiento, y dependiendo de la eficacia del proceso de eliminación de azúcares, no se puede excluir la posibilidad de que los grupos amino de OVA y OM sean glicosilados como consecuencia de la RM, con los subsiguientes modificaciones conformacionales, pudiendo afectar su digestibilidad y alergenicidad. Aunque el efecto del tratamiento térmico sobre las proteínas de huevo ha sido objeto de estudio (Smith, 1964; Donovan y col., 1975; Urisu y col., 1997; Peng y col., 1998; Mine y Zhang, 2002; Photchanachai y col., 2002; Takagi y col., 2003; Talansier y col., 2009), hay pocos trabajos sobre el efecto de la RM en OVA y OM (Kato y col., 1978; Handa y Kuroda, 1999; Rupa y col., 2007; Ilchmann y col., 2010), lo que hace interesante seguir profundizando en el efecto de la glicosilación sobre la alergenicidad de los alérgenos dominantes de la clara de huevo.

3.1.3 Otras interacciones: polisacáridos de interés alimentario.

Las proteínas y los polisacáridos son biopolímeros naturales que pueden ser empleados como ingredientes funcionales, de hecho, las mezclas de proteínas y polisacáridos son usadas frecuentemente con aplicación tecnológica en la industria cosmética, farmacéutica y en la alimentaria. En esta última es común el uso de mezclas

proteína-polisacárido por su capacidad estabilizante para preparar dispersiones alimentarias. Además, los efectos sinérgicos que resultan de la mezcla de estos biopolímeros son de excelente utilidad para la industria alimentaria por la mejora que se consigue en muchos alimentos, por reducir el precio de producción además de crear nuevas nano-, micro- o macroestructuras que mejoran las propiedades organolépticas y reológicas del alimento.

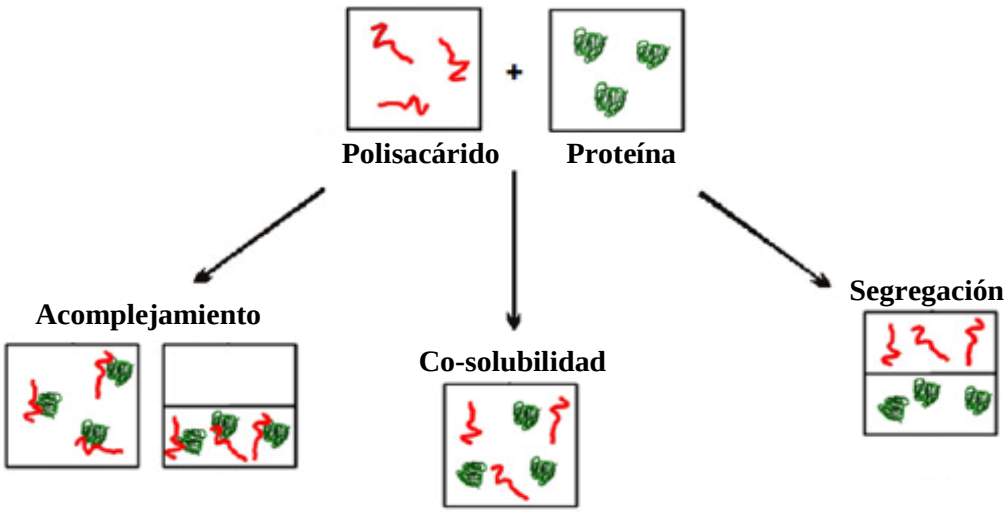


Figura 5. Comportamiento de mezclas proteína-polisacárido. Adaptado de Patino y Pilosof (2011).

En las mezclas de proteínas y polisacáridos, estos pueden unirse mediante enlaces covalentes generando conjugados específicos, fuertes y relativamente estables, como ocurre cuando se da RM. También pueden asociarse por interacciones no covalentes, por ejemplo electrostáticas, hidrofóbicas, puentes de hidrógeno, exclusión estérica, etc (Patino y Pilosof, 2011).

Cuando la proteína se mezcla con el polisacárido pueden darse diversos comportamientos que se hallan ilustrados en la **Figura 5**. Si las mezclas están muy diluidas son estables, dado que la entropía de la mezcla aumenta dándose co-solubilidad de proteínas y polisacáridos. Cuando la concentración de ambos biopolímeros aumenta ocurren fenómenos de segregación o agregación. Las interacciones atractivas entre las proteínas y los polisacáridos pueden dar lugar a la formación de complejos solubles o insolubles. La formación de complejos insolubles provoca una separación de fases, llamada coacervación o separación asociativa. El acomplejamiento de proteínas-polisacáridos es de origen físico y sucede a través de enlaces iónicos, puentes de

hidrógeno o interacciones hidrofóbicas. Sin embargo, cuando los polisacáridos se encuentran cargados, la contribución de fuerzas electrostáticas es predominante. Cuando se dan interacciones repulsivas desfavorables entre polímeros de distinta naturaleza química aumenta la probabilidad de que se dé una exclusión mutua en los alrededores de cada polímero. Normalmente, la incompatibilidad en las mezclas de proteínas-polisacáridos se da a pH superior al pI de las proteínas o en condiciones de elevado estrés iónico. Por tanto, el control del pH y el estrés iónico de la fase acuosa son críticos a la hora de controlar las interacciones proteína-polisacárido en la mezcla (Patino y Pilosof, 2011).

La utilización de polisacáridos solubles de origen vegetal en la industria alimentaria es muy frecuente, debido fundamentalmente a las propiedades tecnológicas de que gozan, destacando la capacidad gelificadora, espesante, emulsionante y estabilizante. Por ejemplo, la presencia de pectinas, goma arábiga, goma guar, alginatos, y carragenanos en alimentos tales como yogur batido, mermeladas, flan, natillas, cremas, helados y puddings es habitual. Además, muchos son componentes naturales de cereales, como es el caso de la xilosa, o de frutas y cítricos, como es el caso de la pectina.

La **pectina** (P) es un componente mayoritario de las paredes celulares de plantas terrestres y algas verdes, donde juega un papel importante en el crecimiento y desarrollo de las mismas (Willats y col., 2001). Los extractos de P son ampliamente usados en la industria alimentaria como ingrediente funcional (E-440) y están presentes en muchos alimentos, verbigracia helados, mermeladas, gelatinas y bebidas lácteas; de hecho, en la dieta occidental se consumen alrededor de 4-5 gramos de P diariamente (Willats y col., 2006). La P es un biopolímero compuesto esencialmente de residuos de ácido D-galacturónico, cuya presencia es responsable del carácter polianiónico de las pectinas, que tienen un pI entorno a 3. Se reconocen en su composición homogalacturonanos no ramificados, rhamnogalacturonanos ramificados de tipo I y rhamnogalacturonanos de tipo II. El grado de metil-esterificación (ME) y los patrones de distribución de metil-ésteres, son importantes para determinar las propiedades funcionales, en especial la gelificadora. En la P con elevado grado de ME las zonas de unión están formadas por entrecruzamiento de cadenas de homogalacturonanos no ramificados por puentes de hidrógeno y fuerzas hidrófobas entre los grupos metoxilo, ambas promovidas por elevadas concentraciones de azúcar y acidez. Este tipo de gelificación hace posible muchos de los usos comerciales de la P, por ejemplo en mermeladas y gelatinas. En la P de bajo ME las zonas de unión están

formadas por entrecruzamientos de calcio y grupos carboxilo libres (Willats y col., 2001; Sorensen y col., 2009).

La **goma arábiga** (G), también llamada goma de acacia, es un polisacárido extraído de la resina de distintas especies de árboles subsaharianos (Acacia senegal y polyacantha) como parte del proceso de cicatrización de estos (gummosis). La G es empleada en la industria alimentaria como aditivo bajo el nombre E-414, o goma de acacia, y tiene un alto contenido en fibra soluble, lo que hace que se empiece a reconocer como fibra alimentaria, aunque fundamentalmente se usa como agente espesante, emulsificante y estabilizante, por ejemplo en siropes y golosinas esponjosas como las "nubes" (Benech, 2008; Ali y col., 2009; Phillips y Phillips, 2011). La G es un polisacárido complejo, ramificado y de elevado peso molecular, de carácter neutro o ligeramente ácido, cuyo cuerpo está compuesto por unidades de D-galactopiranosil unidas por enlace β (1,3). Se ha descrito que la G está compuesta por un 39-42% de galactosa, 24-27% arabinosa, 12-16% de rhamnosa, 15-16% de ácido glucurónico, 1.5-2.6% de proteína, 0.22-0.39% de nitrógeno y un 12.5-16% de humedad, aunque la composición química puede variar dependiendo de la edad de los árboles de los que se obtiene, las condiciones climáticas y la contaminación ambiental (Benech, 2008; Ali y col., 2009; Phillips y Phillips, 2011).

El **xilano** (X) es una de las hemicelulosas más comunes y de los polímeros más abundantes en el reino de las plantas. En las plantas terrestres el X presenta una variedad de cadenas laterales unidas a la principal de β-1,4-D-xilopiranosa e incluyen unidades de α-L-arabinofuranosil y α-D-glucopiranosil. Además, también han sido identificadas en su estructura rhamnosa, xilosa, galactosa, glucosa y una gran variedad de cadenas laterales diméricas y triméricas. Las mayores fuentes de X son la madera de dicotiledóneas y monocotiledóneas no gramíneas y semillas y granos de cereal. La fibra de algunos cereales de plantas agrícolas producidas industrialmente es fuente de X, por ejemplo, la fibra de maíz, que es un producto derivado de la producción de maíz, contiene más de un 50% de heteroxilano altamente ramificado y de gran viscosidad que en la actualidad se llama "*corn fiber gum*" y se usa como goma alimentaria, espesante y adhesivo. Además, entre las propiedades funcionales atribuidas a los polisacáridos se han descrito la capacidad emulsionante y estabilizante, lo que los hace de gran interés para la industria alimentaria (Ebringerova y Hromadkova, 1999; Kacurakova y col., 1999; Ebringerova y Heinze, 2000).

3.1.4 Mezclas polisacárido-proteína: influencia en la alergenicidad.

Si bien no es habitual, se han descrito casos de alergias frente algunos polisacáridos de frecuente uso alimentario. Verbigracia, existen casos de pacientes que desarrollaron alergia ocupacional—que es aquella reacción alérgica frente a alguna sustancia presente en el lugar de trabajo, producida normalmente por inhalación o contacto—a la G (Foetisch y col., 1998; Sander y col., 2006). Además, Foetisch y col. (1998) demostraron que, tras eliminar el contenido proteico de la G, los anticuerpos IgE del paciente eran específicos de epítopos presentes en los carbohidratos. También se han descrito casos de alergia ocupacional frente a P (Rasanen y col., 1998); por ejemplo Kraut y col. (1992) publicaron el caso de un trabajador de la industria de las golosinas que desarrolló alergia frente a P.

Aunque conviene tener presente la posibilidad de que algunos de los polisacáridos empleados en la industria alimentaria sean alergénicos para ciertos individuos, en general, la influencia de los mismos sobre la alergenicidad del alimento proviene de las interacciones que puedan tener con las proteínas alergénicas contenidas en la matriz alimentaria. En ese sentido, se ha visto que los polisacáridos pueden afectar la digestibilidad y alergenicidad de antígenos alimentarios. Mouecoucou y col. (2004) vieron como la presencia de P de bajo grado de ME, G y X reducían la digestibilidad *in vitro* y la unión a IgE de la proteína de cacahuete, hecho que atribuyeron a la formación de complejos entre los productos de hidrólisis del cacahuete y los polisacáridos. Mouecoucou y col. (2007) describieron resultados similares en la β-lactoglobulina de la leche. En la misma línea, recientemente se ha publicado que la P ejerce un efecto protector en la digestión de alérgenos de kiwi, cereza, plátano y manzana, atribuyéndose a su capacidad gelificadora que, al formar geles en el ambiente ácido del estómago, podría disminuir la accesibilidad de las enzimas a la proteína dificultando la pérdida de epítopos durante el proceso digestivo (Polovic y col., 2009). Con estos precedentes, no se puede descartar que en preparados alimentarios en los que coexisten proteínas de huevo y polisacáridos, o incluso al ingerirlos durante la misma comida procedentes de diversos alimentos, se vea afectada la estabilidad de los alérgenos mayoritarios del huevo en el tracto digestivo y, por ende, su capacidad de desencadenar una respuesta inmune.

3.2 Interacción con lípidos: emulsiones

Las emulsiones son sistemas compuestos por dos fases inmiscibles (normalmente agua y aceite) una de las cuales, llamada "fase dispersa", se encuentra dispersa en forma de pequeñas gotas esféricas en la otra fase, denominada "fase continua". El proceso por el cual los dos líquidos inmiscibles pasan a formar una emulsión se conoce como homogeneización. Las emulsiones alimentarias presentan un diámetro de gota de la fase dispersa que va de 0,1 hasta 100 μm (Dickinson y Stainsby, 1982; Dickinson 1992) y pueden ser clasificadas de acuerdo a la distribución de la fase acuosa y oleosa; por tanto, un sistema basado en gotas de agua dispersas en una fase oleosa se conoce como emulsión de agua en aceite W/O (del inglés, *water in oil*), mientras que un sistema en el que hay gotas de aceite dispersas en una fase acuosa se denomina emulsión de aceite en agua o emulsión O/W (del inglés *oil in water*), estando la presente tesis doctoral centrada en las emulsiones O/W por ser de mayor relevancia en el campo que nos ocupa.

En la industria alimentaria este proceso se suele llevar a cabo utilizando dispositivos mecánicos denominados homogeneizadores, que someten a los líquidos a una intensa agitación mecánica. Aunque las emulsiones sean sistemas termodinámicamente inestables es posible formar emulsiones cinéticamente estables durante un periodo de tiempo razonable (días, semanas, meses e inclusive años) mediante la inclusión de sustancias emulsionantes antes de la homogeneización, aunque también son utilizadas a tal efecto sustancias espesantes. Las sustancias emulsionantes más comunes en la industria alimentaria son proteínas anfifílicas, tensioactivos de bajo peso molecular y fosfolípidos, mientras que los agentes espesantes habituales son los polisacáridos. La formación de una emulsión se puede describir como un proceso que comienza con la división de la futura fase dispersa en gotas, continúa con la adsorción de las moléculas de la sustancia emulsionante en la superficie interfacial recién creada y finaliza con la rotura de las gotas en otras de menor tamaño (Halling, 1981).

La resistencia de las emulsiones alimentarias frente a estos mecanismos conservando sus propiedades a lo largo del tiempo se conoce como estabilidad. La estabilidad es una propiedad crucial en las dispersiones alimentarias ya que la percepción del consumidor de la calidad del producto está claramente afectada por la apariencia. En aras de aumentar la estabilidad de la emulsión, el uso de emulsionantes como las proteínas es esencial, ya que éstas generan una serie de interacciones repulsivas (por ejemplo estéricas y electrostáticas) entre las gotas de aceite, y también facilitan la disrupción de las gotas de aceite al disminuir la tensión interfacial, retardan la

coalescencia de las gotas de aceite formando una membrana interfacial, que es resistente a la rotura, alrededor de las gotas de aceite emulsionadas, y se adsorben a las superficies de las gotas de aceite formadas durante la homogeneización de las mezclas aceite, agua y proteínas (McClements, 2004; Malaki-Nik y col., 2011). La película formada es usualmente de carácter anisotrópico y sus propiedades viscoelásticas determinan la resistencia de la emulsión frente a la coalescencia.

Un amplio rango de productos alimentarios se compone de emulsiones O/W llámense mayonesa, leche, crema, sopa y salsa, entre otros (Girard y col., 2002). Las emulsiones alimentarias, al ser sistemas termodinámicamente inestables, tienden a desestabilizarse por diversos mecanismos como la separación gravitacional o cremado que es debida a la diferencia de densidades entre la fase dispersa y la continua; la floculación, que ocurre cuando dos o más gotas se agregan pero mantienen su integridad individual; la coalescencia, que es la unión dos o más gotas de líquido para formar una única gota de mayor tamaño; la maduración de Ostwald, cuando gotas de mayor tamaño crecen a expensas de otras más pequeñas por el transporte de masa de la fase dispersa de una gota a otra a través de la fase continua; y la inversión de fases, cuando el sistema se invierte y la emulsión O/W pasa a ser una emulsión W/O (Taylor, 1995; McClements, 1999).

Los dos pasos críticos en la emulsificación son la ruptura de las gotas de la fase dispersa y su coalescencia, ambas favorecidas por la agitación intensa. La mayoría de los factores del proceso de emulsificación están relacionados con tales aspectos, especialmente con el tamaño de gota, que interesa que sea lo menor posible (McClements, 1999). A tal propósito contribuyen el tipo de emulsificador, que también afecta la eficiencia de ruptura; el uso de emulsionantes, que favorece la rotura de las gotas y conduce, generalmente, a un descenso del tamaño de gota de las emulsiones y previene la recoalescencia de las gotas; la composición y propiedades fisicoquímicas de las fases, el volumen de la fase dispersa; la energía aplicada, la temperatura y tiempo de emulsificación así como el protocolo de emulsificación (Halling, 1981; Braginsky y Belevitskaya, 1996; McClements, 1999).

3.2.1 Efecto de la interacción con lípidos en la alergenicidad

El huevo en su conjunto, así como sus constituyentes (yema y clara), es un ingrediente clave en muchos productos alimentarios de bollería y pastelería, y en la elaboración de mayonesas, salsas y alimentos precocinados. La preparación de salsas con

estabilidad físico-química a largo plazo depende de la presencia de yema de huevo en el sistema. Aunque la yema es un excelente emulsionante, en numerosas ocasiones se usa el huevo entero para la preparación de salsas ya que la clara de huevo, al igual que la yema, está compuesta por proteínas de elevado valor biológico y no exenta de propiedades funcionales (Mine, 1995). Además, la yema de huevo comercial se encuentra contaminada frecuentemente con albumen (Drakos y Kiosseoglou, 2008).

El efecto de algunos tipos de procesado sobre el huevo, en términos de digestibilidad y alergenicidad, ha sido objeto de estudio, sin embargo, es difícil encontrar en la literatura estudios focalizados en la emulsificación de alérgenos de huevo aunque tales efectos se hayan estudiado en otros alérgenos alimentarios obteniéndose resultados relevantes. Por ejemplo, se ha sugerido que el proceso de emulsificación podría conllevar un aumento en la alergenicidad del cacahuete (Sicherer y Sampson, 2007) ya que en regiones como USA y Canadá, donde es habitual el consumo de crema de cacahuete, ha aumentado la prevalencia de alergia a cacahuete en los últimos años. El aumento o descenso de la alergenicidad, como resultado de la interacción del alérgeno con lípidos, puede atribuirse a que la interacción proteja al alérgeno de las condiciones gastrointestinales (Teuber, 2002); este es el caso de la β-lactoglobulina y la β-caseína de la leche, que al estar emulsionadas se digieren *in vitro* con mayor facilidad, aunque algunos de los fragmentos peptídicos formados se vieran protegidos en el caso de la β-caseína (Macierzanka y col., 2009). La α-lactoalbúmina de la leche también se digiere gástricamente menos cuando interacciona con fosfatidilcolina (PC), que es el fosfolípido más abundante de la yema de huevo, además de ser secretado por el estómago, siendo tal efecto atribuido a la inclusión de la proteína en las vesículas de PC (Moreno y col., 2005). También se ha apuntado que las fracciones lipídicas podrían modificar el transporte y/o reconocimiento del alérgeno con el subsiguiente efecto sobre la respuesta inmune (Teuber, 2002), de hecho, se ha descrito que los surfactantes de grado alimentario pueden aumentar el transporte paracelular de alérgenos alimentarios como ocurre con el OM (Mine y Zhang, 2003). Por tanto, la emulsificación de las proteínas de huevo, como parte de una salsa o mayonesa (formas en las que es muy habitual su consumo), podría afectar la resistencia a la digestión gastrointestinal, en base a los cambios conformacionales que sufre la proteína en la interfase, así como su absorción intestinal y, en último término, su potencial alergénico.

4. Aplicación del procesado en el desarrollo de fórmulas terapéuticas

Actualmente, a pesar de la intensa investigación encaminada a encontrar una cura efectiva y segura de las alergias alimentarias, la opción recomendada en personas alérgicas a alimentos continúa siendo la eliminación estricta de la dieta del alimento implicado y/o el uso de fármacos que alivien la sintomatología, pero tan solo se trata de medidas preventivas o paliativas mas no terapéuticas. Además, en algunos casos, debido a la ubiquidad de algunos alimentos, es complicado no estar expuesto a los mismos—como es el caso del huevo— a la par de que algunos estudios apuntan que evitar estrictamente el alimento inicuo podría resultar en un aumento de la sensibilidad al mismo (Sicherer y col., 2001; Allen y col., 2007).

La búsqueda de una cura para las alergias alimentarias es centenaria (Schofield, 1908), sin embargo, la creciente comprensión de los mecanismos subyacentes de la alergia a alimentos, así como la caracterización de alérgenos alimentarios, ha favorecido el desarrollo de diferentes opciones terapéuticas. A la hora de valorar las opciones terapéuticas existentes es importante diferenciar los términos "desensibilización" y "tolerancia", entendiéndose que la desensibilización es una reducción significativa de la respuesta alérgica al alimento desencadenante, mientras que la tolerancia implica la ausencia total de respuesta alérgica tras la exposición al alérgeno causante de la misma.

4.1 Aproximaciones terapéuticas: inmunoterapia oral

Comúnmente, las estrategias terapéuticas se clasifican en tratamientos específicos del alérgeno, como pueden ser la inmunoterapia oral (OIT), inmunoterapia sublingual e inmunoterapia epicutánea, ya sea empleando alérgenos en su forma nativa o alérgenos recombinantes y/o modificados, y en tratamientos no específicos del alérgeno, que incluyen el uso de anticuerpos monoclonales anti-IgE y anti IL-5, preparados de hierbas chinas, terapia con citoquinas, uso de bacterias probióticas y uso de antagonistas de receptores "toll like", entre otros (Nowak-Wegrzyn y Sampson, 2011). Dentro de esta plétora de posibilidades la OIT es la opción más estudiada para tratar la alergia a alimentos por los resultados tan prometedores que se han descrito, además de ser una de las más antiguas (Mine y Yang, 2008).

La OIT consiste en administrar el alimento o ingrediente ofensivo en dosis que van aumentando gradualmente para desensibilizar al individuo y finalmente inducir tolerancia oral. Alcanzar la desensibilización, como indicábamos con anterioridad, no significa la cura total y la protección alcanzada va depender de la ingesta regular del

alimento ofensivo, mas si ésta es interrumpida el efecto protector podría perderse o reducirse notablemente. Alcanzar la tolerancia oral implica el desarrollo de células T-reg y la reducción de la respuesta pro-alérgica Th-2 seguida finalmente de un estado de anergia, estando el paciente completamente curado (Uermosi y col., 2010). La fase de escalado generalmente se da en un ambiente controlado para mayor seguridad el paciente pero, una vez alcanzada la desensibilización, la fase de mantenimiento se suele hacer sin supervisión médica. Basándonos en los estudios publicados hasta la fecha existen distintos patrones de respuesta a la OIT y quedan interrogantes que es necesario resolver antes de que la OIT sea empleada más allá del ámbito experimental (Nowak-Wegrzyn y Sampson, 2011) (**Figura 6**).

Figura 6. Cuestiones pendientes de resolver relativas a la OIT.

Por ejemplo, a pesar de los resultados esperanzadores que se han obtenido, son pocos los estudios que han establecido la reactividad inicial del paciente, lo que haría prever una terapia exitosa, o que han incluido un grupo placebo control. Normalmente, alrededor de un 10-20% de los pacientes no consigue alcanzar la desensibilización durante el periodo inicial y abandona los protocolos debido a la aparición de reacciones alérgicas notorias. En torno a un 10-20% son incapaces de completar la fase de mantenimiento alcanzando únicamente una desensibilización parcial. Por tanto, un 50-75% de los pacientes consiguen alcanzar y tolerar la dosis de mantenimiento. La mayoría de los niños toleran más de 5g del alimento alergénico durante la terapia, pero es

necesario identificar marcadores que contribuyan a determinar si la desensibilización parcial desembocará en tolerancia alargando la OIT. También se desconoce si el fracaso en la desensibilización está asociado con un fenotipo más severo y permanente de alergia a alimentos, en contraposición con los casos donde se induce la tolerancia oral de forma espontánea, que están asociados con un fenotipo clínico de alergia pasajera con más probabilidades de resolver la alergia alimentaria. Además, aunque hay algunos indicadores inmunológicos asociados con una inmunoterapia oral exitosa, como son una reducción de los anticuerpos específicos IgE, la inducción de IgG4/IgG2a específica, aumento de la respuesta Th-1 con disminución de la Th-2 y desarrollo de un estado de anergia en células T (Burks y col., 2008), los mecanismos inmunológicos que llevan a la cura no se conocen del todo y quedan dudas por resolver, verbigracia el papel que ejercen los anticuerpos IgA específicos en la mucosa gastrointestinal (Fagarasan y col., 2010) o el controvertido papel de las IgG específicas y las distintas clases de isotipos en la respuesta alérgica (Uermosi y col., 2010).

4.2 Inmunoterapia oral para alérgicos a huevo

La aplicación del procesado de alimentos en la preparación de fórmulas para OIT es una realidad palpable y asaz frecuente en pacientes alérgicos a huevo. Actualmente, la opción más valorada es la elaboración de preparados que contengan huevo tratado térmicamente (**Figura 7**). Como se ha mencionado, el calentamiento intenso del huevo disminuye la alergenicidad debido a la rotura de epítopos conformacionales, que afecta a aquellos alérgenos de huevo termolábiles que contengan epítopos espaciales en su estructura, como es el caso de la OVA. A partir del procesado térmico del huevo se pueden obtener fórmulas relativamente seguras para OIT y con poder terapéutico. De este modo, Urisu y col. (1997) vieron que la mitad de los pacientes alérgicos a huevo toleraron clara de huevo calentada a 90ºC durante 60 minutos y Lemon-Mule y col. (2008) comprobaron que el 74% (87) de los pacientes toleraron la ingesta de huevo calentado como ingrediente de un bollo (175ºC, 30 min) o un gofre (260ºC 3 min). Resulta interesante el hecho de que, aunque se trate de estudios distintos, más pacientes tolerasen el huevo calentado que la clara calentada, lo que puede ser atribuido a que el potencial alergénico del huevo se halla mayormente concentrado en la clara. Además, mientras que la OVA, OT y LYS son termolábiles, lo cual no quita para que contengan epítopos secuenciales en su estructura que resistan el tratamiento térmico, el OM es termoestable y reconocido como inmunodominante. Siguiendo está línea, Urisu y col. (1997) fueron

pioneros en la elaboración de una fórmula de OIT para alérgicos a huevo basada en huevo calentado con bajo contenido en OM, siendo tal preparado tolerado por un 95% de los pacientes. Este preparado hipoalergénico ha demostrado tener propiedades terapéuticas; recientemente se ha visto que un 48% (28) y un 44% (24) de pacientes alérgicos a huevo fueron desensibilizados tras un mes o dos respectivamente de OIT (Urisu y col., 2008; Urisu y col., 2010).

La utilización de clara de huevo calentada con bajo contenido en OM, así como otros preparados de huevo calentado, están siendo objeto de estudio (**Figura 7**) para ser empleados en la OIT de pacientes alérgicos a huevo, sin embargo, como señalábamos antes, es necesario profundizar en los mecanismos inmunológicos involucrados en la OIT antes de poner a punto los protocolos de OIT y que estos puedan ser aplicados de forma rutinaria. Para tal menester, a parte de estudios clínicos, la utilización de modelos animales, como los roedores, son de gran ayuda. El uso de ratones ofrece numerosas ventajas por la posibilidad de intervención en las distintas cepas (transgénicos, *knock-out*, etc.), pequeño tamaño, fácil manejo, elevado índice de natalidad, gran diversidad genética y la existencia de modelos optimizados para el estudio de la respuesta alérgica tardia, inmediata, rinitis, anafilaxis, etc., ofreciendo un abanico de posibilidades experimentales que de cualquier modo sería inviable en humanos (Zubeldia, 2001).

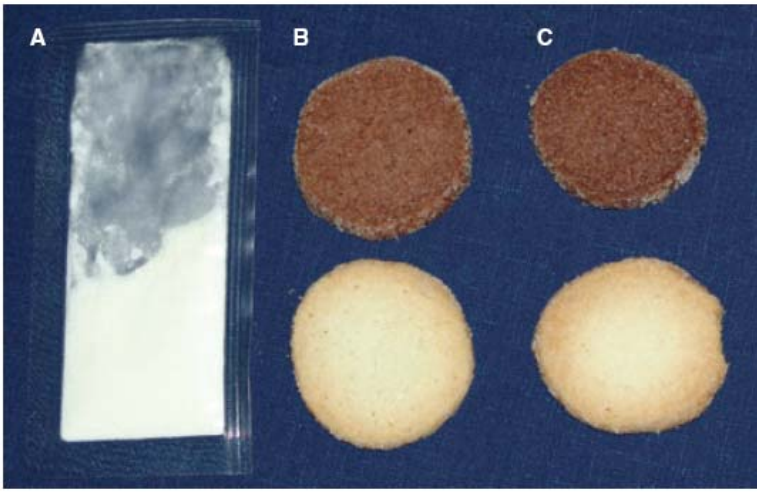


Figura 7. Preparados habituales para OIT en alérgicos a huevo: a) liofilizado de clara de huevo; b) galleta de huevo calentado con bajo contenido en OM; c) galleta de clara de huevo calentada. Tomado de Benhamou y col. (2010)

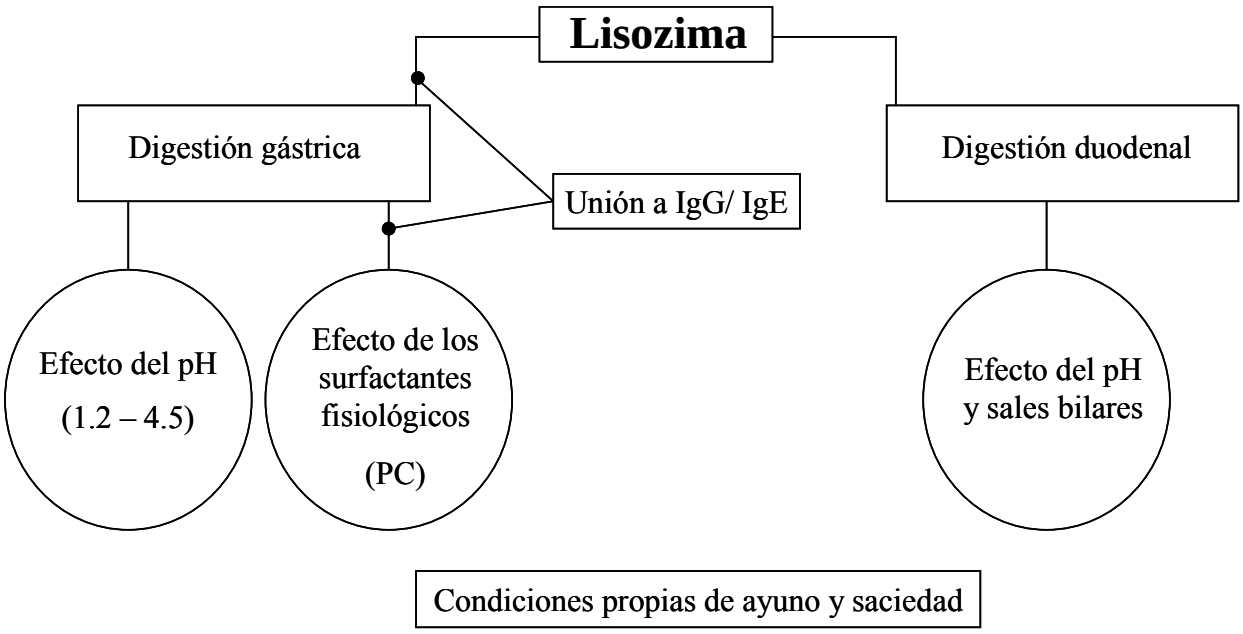
OBJETIVOS Y PLAN DE TRABAJO

OBJETIVOS Y PLAN DE TRABAJO

Actualmente, a pesar de los avances en inmunología y de que la inmunoterapia oral está convirtiéndose en una alternativa sólida para tratar las alergias alimentarias, la opción comúnmente recomendada y más segura, en personas alérgicas a alimentos, es eliminar de la dieta el alimento desencadenante. No obstante, en el caso de la alergia al huevo, es tarea ardua dada la omnipresencia de compuestos derivados del huevo en productos cocinados o manufacturados, lo que frecuentemente da lugar a exposiciones inadvertidas, que pueden tornarse peligrosas si se desencadenan respuestas anafilácticas.

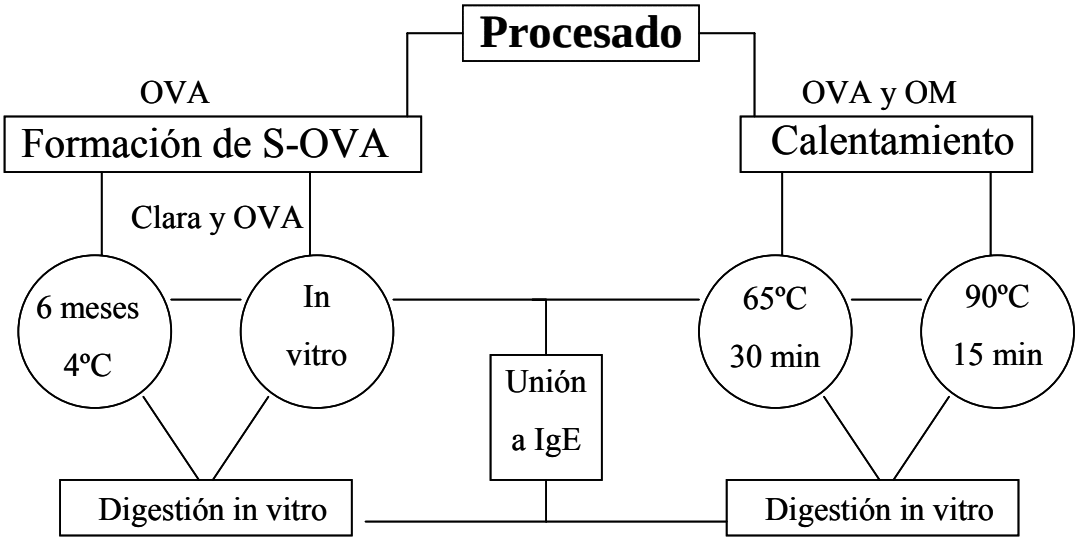
La alergenicidad depende en gran medida, pero no exclusivamente, de la resistencia de los alérgenos al procesado y a las enzimas digestivas, consiguiendo llegar intactos al intestino. Dicha resistencia se atribuye a varios factores, entre los que destaca la estructura del propio alérgeno, su flexibilidad y la accesibilidad de los lugares de acción de las enzimas, que puede sufrir modificaciones durante el procesado y el almacenamiento de los alimentos. Además se ha demostrado que aspectos tales como la absorción a interfases o la presencia de surfactantes fisiológicos durante la digestión pueden jugar un papel fundamental en la digestibilidad y en la inmunogenicidad de algunas proteínas. La comprensión de las propiedades de las proteínas alergénicas exige tener en cuenta la diversidad de ingredientes y operaciones que se emplean en tecnología de alimentos, así como la complejidad de los procesos digestivos. En la investigación sobre alergias alimentarias, la mayoría de los trabajos se centran en proteínas puras, pero un aspecto crucial es la forma en que estas proteínas se digieren como parte de matrices complejas.

La LYS es una ovoproteína ampliamente utilizada debido a su potente actividad antibacteriana. No obstante, se sabe poco sobre su comportamiento en condiciones gastrointestinales, o sobre la alergenicidad remanente de los digeridos, encontrándose incluso resultados contradictorios en la bibliografía. Aun no siendo considerada tan alergénica como la OVA o el OM, su amplio uso en preparados farmacológicos y alimentos hace interesante su estudio empleando sistemas de digestión *in vitro* que reproduzcan condiciones fisiológicas. Por lo tanto, nuestro **primer objetivo fue investigar el comportamiento de la LYS bajo diferentes condiciones gastrointestinales experimentales y evaluar la alergenicidad *in vitro* de los digeridos,** siguiendo el plan de trabajo ilustrado en el esquema 1.



Esquema 1. Plan de trabajo seguido para investigar el comportamiento de la LYS bajo diferentes condiciones gastrointestinales experimentales, que se dan en estados de ayuno, saciedad o en estados intermedios, y evaluar la alergenicidad *in vitro* de los digeridos.

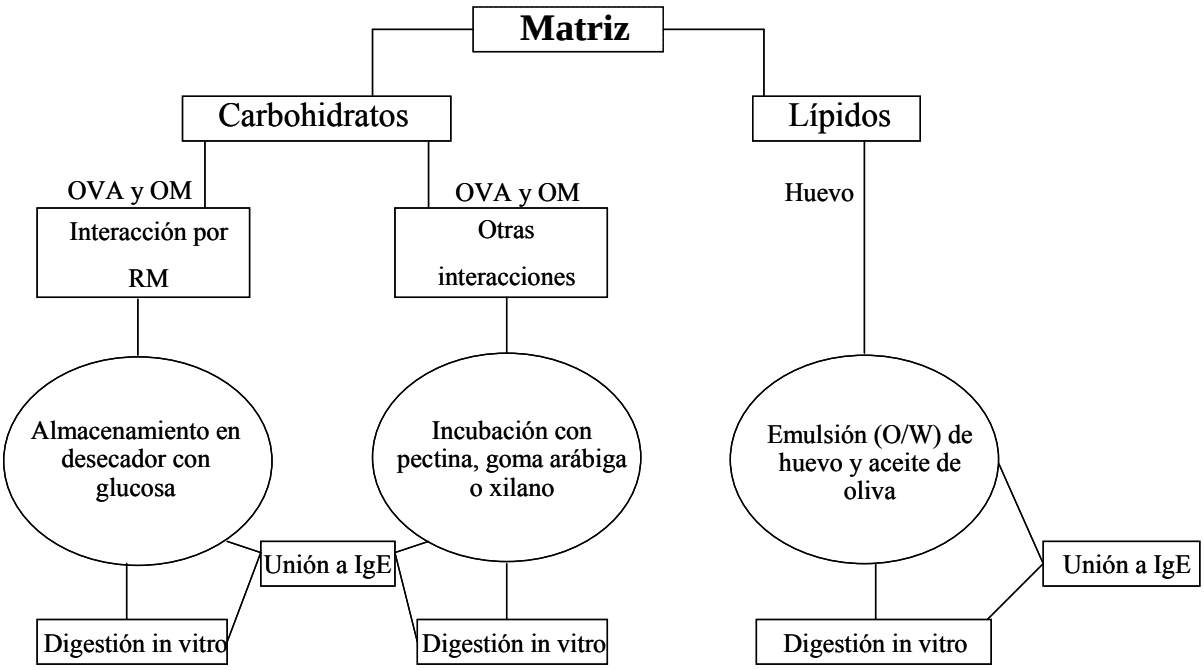
El almacenamiento de los alimentos hasta su consumo puede dar lugar a modificaciones estructurales. Este es el caso de la OVA que, con el tiempo, se transforma una forma más termoestable, llamada S-OVA. Aunque la S-OVA ha sido investigada, su estudio se ha abordado generalmente desde una perspectiva industrial relacionada con la pérdida de las propiedades funcionales del huevo, sin haberse evaluado su digestibilidad y alergenicidad. Por otra parte, el tratamiento térmico, parte habitual del procesado, puede inferir cambios estructurales importantes en las proteínas, como desdoblamiento o agregación, que podrían afectar tanto a su digestibilidad como a su capacidad de desencadenar una respuesta alérgica. Por ejemplo, el calentamiento puede provocar entrecruzamientos covalentes en la estructura, estabilizándola y haciéndola más resistente al proceso digestivo, o desdoblar la proteína y que se vea facilitada la acción de las enzimas digestivas. Aunque el efecto del tratamiento térmico en proteínas de huevo ha sido objeto de estudio, no siempre se ha evaluado cómo afectaba éste a la digestibilidad de las proteínas y/o a la alergenicidad de los digeridos. Basándonos en estas evidencias planteamos el **segundo objetivo: estudiar el efecto del almacenamiento, en concreto la formación de S-OVA, así como el del tratamiento térmico de la OVA y el OM, sobre su digestibilidad y alergenicidad *in vitro,*** siguiendo el plan de trabajo ilustrado en el esquema 2.



Esquema 2. Plan de trabajo seguido para estudiar el efecto del almacenamiento y procesado (formación de la S-OVA y tratamiento térmico de la OVA y el OM) sobre su digestibilidad y alergenicidad *in vitro*.

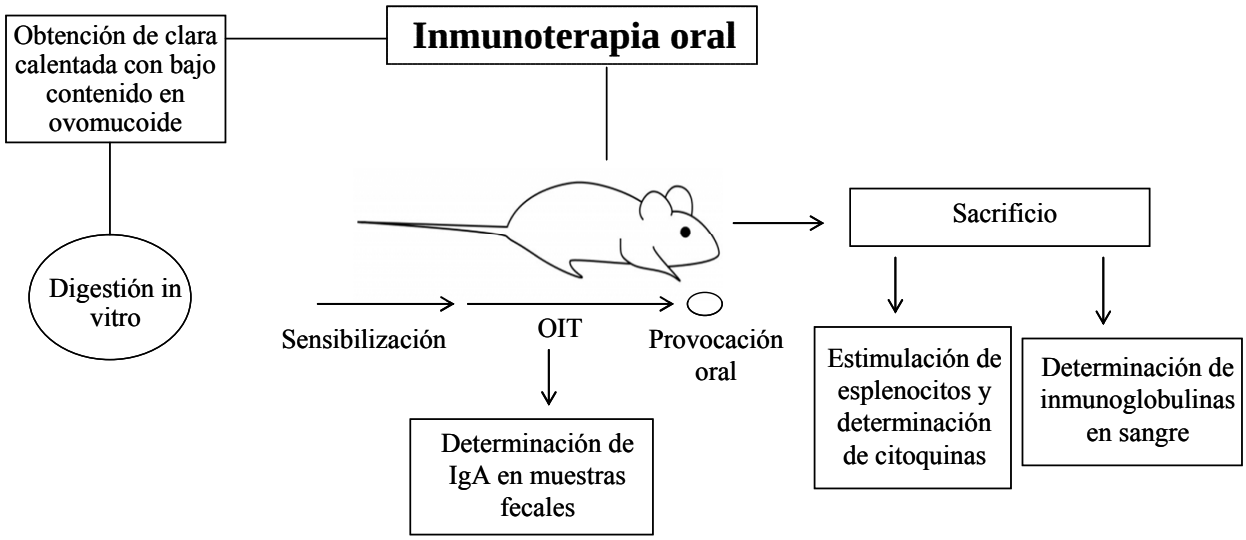
La incorporación de proteínas en estructuras coloidales, tales como geles, espumas o emulsiones y, en general, las interacciones que puedan darse entre los alérgenos y otros componentes que se hallen en la matriz en la que son consumidos, o que se ingieran conjuntamente con ellos, pueden modificar la forma en la que son digeridos y la inmunorreactividad de los mismos. Las interacciones más frecuentes de proteínas con otros componentes de la matriz se dan con carbohidratos y lípidos. La glicosilación, vía RM, ocurre entre azúcares reductores, y la clara de huevo contiene hasta un 4% de glucosa respecto al contenido en sólidos, y proteínas susceptibles con grupos amino libres. También pueden darse interacciones no covalentes entre proteínas y carbohidratos. Por ejemplo, el uso frecuente de polisacáridos, como P, G o X en la industria alimentaria, hace factible que interaccionen electrostáticamente o mediante puentes de hidrógeno con alérgenos de huevo, ya sea en los alimentos que contienen ambos o durante la digestión. Además de las interacciones con carbohidratos, es reseñable la interacción con lípidos, dado que el huevo es frecuentemente consumido en una matriz lipídica, como puede ser una mayonesa o salsa. La adsorción de proteínas a las interfases puede limitar la exposición de la porción adsorbida a las enzimas digestivas aunque, de modo general, el aumento de flexibilidad que caracteriza a la adsorción aumenta la proteolisis. Si bien existen muchos estudios referidos a emulsiones de huevo, la gran mayoría se centran en sus propiedades tecnológicas, sin

explorar el comportamiento de tales emulsiones en cuanto a digestibilidad y alergenicidad. Por tanto, el **tercer objetivo fue investigar el efecto de la matriz, ya sea rica en carbohidratos (glucosa o polisacáridos) o en lípidos (emulsión O/W) sobre la digestión de alérgenos de huevo y la alergenicidad *in vitro* de los digeridos resultantes,** de acuerdo al plan de trabajo detallado a continuación (esquema 3):



Esquema 3. Plan de trabajo seguido para investigar cómo influyen las interacciones con algunos componentes de la matriz alimentaria en la digestibilidad y alergenicidad *in vitro* de alérgenos de huevo.

La utilización de fórmulas de huevo procesado para OIT en pacientes alérgicos a huevo es habitual, destacando el preparado de clara de huevo calentada con bajo contenido en OM porque, además de tener propiedades inmunoterapéuticas, es hipoalergénico. No obstante, los mecanismos implicados en la desensibilización e inducción de tolerancia oral no se conocen completamente, siendo necesario profundizar en ellos, así como identificar marcadores que permitan evaluar los efectos terapéuticos asociados con el avance de la OIT. Con tales precedentes, el **cuarto objetivo fue investigar los mecanismos inmunomoduladores mediante los cuales ejerce su efecto una fórmula de clara de huevo procesada (tratada térmicamente y con bajo contenido en OM) en un modelo de ratón alérgico a huevo,** siguiendo el plan de trabajo reflejado en el esquema 4.



Esquema 4. Plan de trabajo seguido para estudiar los mecanismos inmunomoduladores de la fórmula de huevo procesada (tratada térmicamente y con bajo contenido en OM) en un modelo de ratón alérgico a huevo.

RESULTADOS

RESULTADOS

Los resultados obtenidos serán presentados en forma de artículos, ya sea publicados o en preparación, ordenados de la siguiente forma:

1. Jiménez-Saiz R, Martos G, Carrillo W, López-Fandiño, Molina E. Susceptibility of lysozyme to *in-vitro* digestion and immunoreactivity of its digests (2011). *Food Chemistry* 127: 1719-1726.

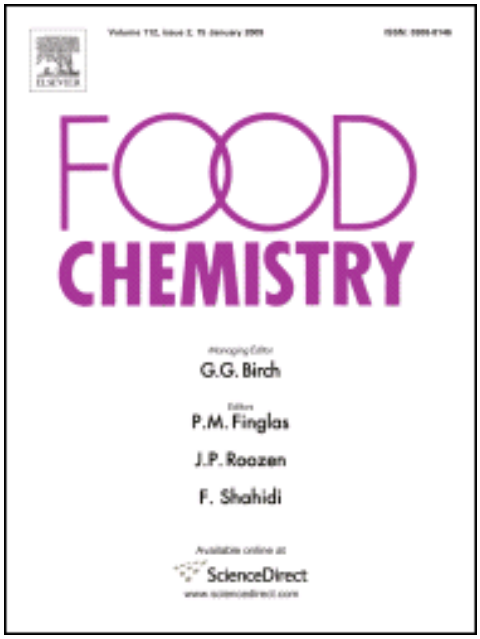
2. Jiménez-Saiz R, Pineda-Vadillo C, López-Fadiño R, Molina E. Human-IgE binding and *in vitro* digestion of S-OVA. *Food Chemistry (Enviado).*

3. Jiménez-Saiz R, Belloque J, Molina E, López-Fandiño R (2011). Human immunoglobulin (Ig) E binding to heated and glycated ovalbumin and ovomucoid before and after *in vitro* digestion. *Journal of Agricultural and Food Chemistry* 59: 10044-10051.

4. Jiménez-Saiz R, López-Expósito I, Molina E, López-Fandiño R. Intestinal stability of egg allergens in the presence of polysaccharides. *Food Hydrocolloids (Enviado).*

5. Jiménez-Saiz R, Ruíz-Henestrosa VMP, López-Fandiño R, Molina E. *In vitro* digestibility and allergenicity of emulsified hen egg. *Food Research International (Enviado).*

6. Jiménez-Saiz R, Rupa P, Mine Y. Immunomodulatory effects of heated ovomucoid-depleted egg white in a Balb/c mouse model of egg allergy (2011). *Journal of Agricultural and Food Chemistry* 59: 13195-13202.



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Susceptibility of lysozyme to *in-vitro* digestion and immunoreactivity of its digests

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ABSTRACT

This paper examines lysozyme (LYS) behaviour upon *in-vitro* digestion, mimicking different conditions in the stomach and intestine, and assessing the effect of natural surfactants, such as phosphatidylcholine (PC) or bile salts (BS), on hydrolysis and residual immunogenicity of the digests. The hydrolysis pattern of LYS was compared to that of α-lactalbumin (LA). Hydrolysis of LYS only occurred at low pH. PC slightly increased its resistance to pepsinolysis. A similar behaviour was found for LA. Circular dichroism revealed that the more rigid structure of LYS, as compared with that of LA, could protect it from proteolysis at acidic pH and fluorescence spectra suggested that, at acidic pH, both proteins associated to PC films. The gastric digests of LYS showed high IgE-binding capacity using sera from egg-allergic patients. On the other hand, it was found that LYS precipitated under conditions that simulated a duodenal environment, mainly due to the presence of BS.

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1. Introduction

Lysozyme (LYS) is, together with lactoferrin, one of the most extensively studied antibacterial milk proteins (López-Exposito & Recio, 2006). LYS is present in milk from different species, but also in many biological fluids and tissues, such as human milk, tears and saliva. Its bactericidal effect partially depends on its lytic activity on the cell wall of Gram-positive microorganisms. Many attempts have been made to broaden its antimicrobial activity to include Gram-negative bacteria, such as the use of thermal treatments to partially denature the protein, the linkage of hydrophobic ligands to increase its hydrophobicity or the conjugation to polysaccharides through controlled Maillard reaction (Pellegrini et al., 1997). In addition to conformational changes, proteolysis of LYS has been shown to produce peptides able to induce a non-enzymatic bacterial inactivation and are, therefore, active against Gram-positive and Gram-negative bacteria (Ibrahim, Inazaki, Abdou, Aoki, & Kim, 2005; Mine, Ma, & Lauriau, 2004). Other biological functions of LYS, such as immunomodulatory, antiviral and anti-inflammatory, have been reported (Lesnierowski & Kijowski, 2007).

It can be presumed that milk LYS can act physiologically as a bactericidal agent, although the existence of a definite protective role is not yet known. In fact, the high level of LYS in human milk (which is 100 times higher than that of bovine milk, which contains from 0.13 to 0.32 mg/L of LYS) could be of relevance (Fox & Kelly, 2006). In any case, exogenous LYS is widely added to several cheese varieties to prevent the growth of *Clostridium*, which causes off-flavours and late blowing and its use, in combination with minimal processing techniques, has been proposed to extend the shelf life of milk and dairy products (Sobrino-López & Martín-Belloso, 2008).

Hen egg white (probably the richest source of LYS, containing 1–3 g/L) is the main commercial source of LYS used to maintain the quality of food and pharmaceutical products. Members of the LYS superfamily are related proteins, with similar three-dimensional structures but different amino acid sequences, comprising from 123 to 129 amino acids. Egg-white LYS (Gal d 4, with mass of 14.3 kDa, 129 amino acid residues and four disulphide bridges) has been extensively studied as a model protein for structure and biological properties of LYS from other sources. However, LYS is also a major allergen of egg white, although its allergenic potential has not been studied in depth and no relevant epitopes have been identified as yet. Clinical reactions to egg LYS have been described and anti-LYS IgE antibodies are frequently found in egg allergic patients as markers of sensitisation (Mine & Yang, 2008). In fact, the frequent presence of LYS as an additive in dairy products poses a risk to allergic subjects (Iaconelli et al., 2008).

It is generally accepted that resistance to digestion is a common feature to food allergens, although it has also been shown that proteolytic fragments produced during digestion may bind IgE and induce allergic responses in sensitised individuals. Besides, in the case of LYS, digestion can be a physiological process to release peptides that contribute to its *in vivo* defence role. However, available information related to the basis of the resistance of this

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protein to digestion is limited and, sometimes, contradictory. This can be attributed to the fact that *in-vitro* digestion models use inappropriate amounts of proteases or one-step digestion models, disregarding the complex gastric and duodenal composition and the interactions of proteins with other components, such as lipids (Moreno, 2007).

This paper examines LYS behaviour upon *in-vitro* digestion, mimicking different conditions in the stomach and upper intestine, and assessing the effect of natural surfactants, such as phosphatidylcholine (PC) or bile salts (BS), on hydrolysis and residual immunogenicity of the digests. The structural characteristics of LYS that could play a role in its susceptibility to proteolysis under different conditions were studied and compared to those of its homologous protein bovine milk α-lactalbumin (LA), which is more susceptible to proteolysis. Because of its broader availability, hen-egg LYS, which shows 60% homology with human milk LYS, was used. The results obtained regarding egg-white LYS behaviour towards digestion can be relevant in understanding its allergenic potential, of importance when added to dairy products, and the contribution of physiological processes to its *in-vivo* defence role that could be common to other members of the LYS family.

2. Materials and methods

2.1. Gastric and Duodenal digestions

LYS (L2879, chloride form from chicken egg white Grade VI, ~60000 units/mg protein, EC 3.2.1.17, Sigma–Aldrich, St. Louis, MO) was subjected to *in-vitro* gastric digestions at 5 mg/mL final concentration. The digestions were performed in simulated gastric fluid (SGF, 0.35 M NaCl) at different pHs: 1.2, 2.0, 3.2, 4.0 and 4.5, at 37 °C for 120 min, with porcine pepsin (EC 3.4.23.1, 3440 U/mg protein, Sigma–Aldrich) at an enzyme: substrate ratio (E:S) of 1:20, wt/wt (172 U/mg), considered as a physiological ratio (Moreno, Mellon, Wickman, Bottrill, & Mills, 2005a).

Gastric digestions in the presence of the natural surfactant phosphatidylcholine (PC, Sigma–Aldrich), were also assessed. Phospholipid vesicles were prepared by dissolving PC in SGF pH 2.0 (9.58 mg/mL), following Martos, Contreras, Molina, and López-Fandiño (2010).

At least three replicates of each digestion assay were performed and compared by HPLC and SDS–PAGE to ensure the repeatability of the results. Aliquots were taken at different time points, up to 120 min, for analysis. The digestions were stopped by mixing with the SDS-sample buffer for SDS–PAGE analyses, whereas for RP-HPLC and inhibition ELISA, the pH was raised to 7.0 with ammonium bicarbonate to irreversibly inactivate pepsin and, after 10 min of equilibration, the digestion mixtures were centrifuged at 10000*g* and 20 °C for 10 min. The protein concentration of the supernatants was determined by the Kjeldahl method.

Bovine α-lactalbumin (LA, Sigma–Aldrich) was also submitted to *in-vitro* gastric digestions without and with PC under the same conditions. In all cases, at least triplicate digestions were conducted.

Duodenal digestions were performed as previously described (Martos et al. 2010; Moreno, Mackie, & Mills 2005b; Moreno et al., 2005a) on the supernatants of the 60-min gastric digests re-adjusted to pH 6.5, with the addition of: a 0.125 M bile salt (BS) mixture containing equimolar quantities of sodium glycodeoxycholate and sodium taurocholate (Sigma–Aldrich) (6.15 mM final concentration of each salt); 1 M $CaCl_2$ (7.6 mM final concentration); pancreatic porcine lipase (Type VI-S, 111000 U/mg protein, Sigma–Aldrich), at an E:S of 1:3895, wt/wt (28.5 U/mg); pancreatic porcine colipase (Sigma–Aldrich), at an E:S of 1:895, wt/wt; pancreatic bovine trypsin (type I, 10100 U/mg protein, Sigma–Aldrich), at an E:S of 1:238, wt/wt (42.5 U/mg); and pancreatic bovine α-chymotrypsin (type I–S, 58.3 U/mg protein, Sigma–Aldrich), at an E:S of 1:115 wt/wt (0.52 U/mg) in 20.3 mM Bis–Tris. The reactions were carried out at 37 °C for either 30 or 60 min and stopped by heating at 80 °C for 5 min.

2.2. Solubility experiments

The solubility of LYS was examined in SGF at different pHs (1.2, 2, 3.2, 3.6, 3.9, 4.7, 5.1, 6.2, 6.4, 6.8, 7.2 and 7.7) as well as in 10 mM phosphate buffer. Solubility of the intact protein was also assessed in 7.6 mM $CaCl_2$ and 20.3 mM Bis–Tris under simulated duodenal conditions typical of a fasted state (3 mM of each BS and 2.4 mM PC, pH 7.0), of a fed state (9 mM of each BS and 7.2 mM PC, pH 6.0) and of an intermediate state (6 mM of each BS and 4.9 mM PC, pH 6.5) (Kostewicz, Brauns, Becker, and Dressman, 2002; Kaukonen, Boyd, Porter, & Charman, 2004). This last condition was similar to the medium used for duodenal digestion. Solubility was determined by SDS–PAGE and/or RP-HPLC analysis following centrifugation (10000*g*, 20 °C, 10 min).

2.3. SDS–PAGE

SDS–PAGE of the gastric digests was performed using Tris-Tricine ready gels with 16.5% acrylamide (Bio-Rad Laboratories, Hercules, CA). Samples were diluted in Tris-Tricine sample buffer (Bio-Rad). Electrophoresis was carried out at 100 V, for 3 h, at room temperature (RT), in Tris-Tricine SDS running buffer (Bio-Rad). Gels were fixed in a 40% methanol and 10% (wt/vol) acetic acid solution, followed by staining with Coomassie Blue G-250 (Bio-Rad). The kaleidoscope pre-stained standards (Bio-Rad) containing myosin (198 kDa), β-galactosidase (125 kDa), BSA (88 kDa), carbonic anhydrase (37 kDa), STI (31 kDa), LYS (17 kDa) and aprotinin (7 kDa) were used.

SDS–PAGE of the duodenal digests was performed using PhastSystem equipment (Pharmacia, Uppsala, Sweden) using PhastGel Homogeneous 20 polyacrylamide gels and PhastGel SDS-Buffer Strips (Pharmacia) and following the manufacturer's separation and Coomassie staining conditions. The low molecular weight calibration kit for SDS electrophoresis (GE Healthcare, Uppsala, Sweden) containing phosphorylase B (97 kDa), BSA (66 kDa), OVA (45 kDa), carbonic anhydrase (37 kDa), trypsin inhibitor (20.1 kDa), and LA (14.4 kDa), was used.

2.4. RP-HPLC

LYS hydrolysates, at a concentration of 2.4 mg/mL, were analysed using a Hi-Pore® RP-318 (250 × 4.6 mm i.d.) column (Waters, Milford, MA) in a Waters 600 HPLC system. Solvent A was 0.37% (vol/vol) trifluoroacetic acid (Scharlau Chemie, Barcelona, Spain) in double-distilled water and solvent B was 0.27% (vol/vol) trifluoroacetic acid in HPLC-grade acetonitrile (Lab-Scan, Gliwice, Poland). The chromatographic conditions were as in Martos et al. (2010). Detection was at 220 nm and data were processed by using Empower 2 Software (Waters).

2.5. Circular dichroism spectroscopy

CD spectra were recorded in the far (195–260 nm) and near (250–350 nm) UV regions, using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan) as described in Martos et al. (2010). LYS and LA were dissolved at 0.2 mg/mL for the analysis in the far-UV region and at 2 mg/mL for the near-UV region, either in SGF (pH 2.0) or in 10 mM phosphate buffer (pH 7.0). PC was added at 0.184 mg/mL or 1.84 mg/mL, respectively, to maintain the protein/phospholipid ratio used during digestion. Buffer blanks were subtracted from each CD spectrum.

2.6. Fluorescence spectroscopy

The interaction between either LYS or LA and PC was studied by fluorescence spectroscopy based on Barbana et al. (2006). Fluorescence spectra between 300 and 380 nm (excitation: 280 nm) were recorded at room temperature on a Shimadzu RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan). The binding of PC was measured by following the increase in protein fluorescence. The procedure used for titration of LYS and LA was as described in Martos et al. (2010).

2.7. IgG- and IgE-binding by inhibition ELISA

The antigenicity of LYS and its gastric hydrolysates was evaluated by inhibition ELISA using commercial IgG antibodies. Single wells of polystyrene microtitre plates (Corning, Cambridge, MA) were coated with 10 μg/mL of LYS solution in 0.01 M PBS, pH 7.4, and incubated overnight at 6 °C. Afterwards, the plates were washed three times with PBS-Tween 20 (PBST) 0.05% using a Microplate Washer (Nunc, Roskilde, Denmark) and PBST 2.5% was used as saturating agent, to avoid non-specific binding. Then, the plate was blocked for 4 h at RT and washed once.

Serial dilutions of each sample (not less than 10) were incubated (1:1, vol/vol) at RT for 2 h, with horseradish peroxidase (HRP) conjugated-polyclonal anti-LYS raised in rabbit (Abcam, Cambridge, UK), previously diluted 1:10000 in PBST 0.05%, and 50 μL were added to each well. After 2 h of incubation at RT, the plate was washed three times and 50 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (ready-to-use solution; Sigma–Aldrich) were added to each well. Finally, the reaction was stopped with 0.5 M sulphuric acid and the absorbance was measured at 450 nm. A negative control without antibody (native protein in PBST 0.05%) and positive controls (antibody diluted in PBS) were included in each plate.

For human IgE-binding, three individual serum samples from children with clinically allergic symptoms to egg white proteins were used. The sera were collected from the Maternal and Child Gregorio Marañon Hospital (Madrid, Spain). The patients had specific seric IgE levels towards egg white higher than 100 kU/L, as determined by CAP (GE HealthCare). The procedure described above was followed but, in this case, serial dilutions of each sample (not less than 10) were incubated at RT for 2 h with the patient's sera (1:1, vol:vol), previously diluted in PBST, and 50 μL were added to each well. After 2 h of incubation at RT, the plate was washed three times and 50 μL of HRP-conjugated rabbit anti-human IgE (DakoCytomation, Glostrup, Denmark), diluted 1:1000 in PBST 0.05%, were added per well and incubated for 1 h at RT. Then the plate was washed three times and the tyramide-biotin and streptavidin-HRP amplification system was used following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Waltham, MA). Finally, the plate was washed four times and TMB was used as substrate. The reaction was stopped with 0.5 M sulfuric acid and the absorbance was measured at 450 nm. A negative control without serum (native protein in PBST) and positive controls (sera diluted in PBST 0.05%) were included in each plate.

Both IgG- and IgE-binding results were statistically processed. A non-linear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoid curve of inhibition dose-response with variable slope, from which the IC_{50} (the protein concentration that binds 50% of either IgG or seric IgE) was obtained with the program GraphPad PRISM 4 for Windows (GraphPad software, San Diego, CA). The IgG- and IgE-capacities of the digests were expressed as percentages of the IC_{50} of the intact protein (as means ± standard errors for $n = 3$). Significant differences ($p < 0.05$) were evaluated by one-way analysis of variance.

3. Results and discussion

3.1. Effect of the pH on the gastric digestion of LYS in vitro

Fig. 1 shows the SDS–PAGE pattern of LYS digested with pepsin at an E:S of 1:20 (wt/wt), after different hydrolysis times at different pHs, in order to simulate different conditions in the stomach, from the lowest values typical of a fasted state, to higher values characteristic of a fed state or of immature stomach functions. Hydrolysis of the protein only occurred at pH 1.2 and 2.0, with the formation of degradation products of less than 6 kDa that resisted pepsin action for at least 2 h of digestion. Interferences arising from the autodigestion of pepsin were also assessed, but no additional bands were detected in the electrophoretic separations at this enzyme concentration.

LYS had disappeared before 60 min of digestion at pH 1.2, while there still was unhydrolysed protein after 120 min at pH 2.0, a pH that prevails in the fasted stage of the stomach of healthy adults. The presence of a partially folded intermediate of LYS, characterised by a significant secondary structure, exposure of non-polar clusters and a disrupted tertiary structure, has been reported at very low pH values (1.5) (Polverino de Laureto, Frare, Gottardo, Van Dael, & Fontana, 2002). This increased flexibility could be responsible for its increased susceptibility to digestion at pH 1.2. The pH had a very important effect on LYS hydrolysis, compared to pepsin, which exhibits its optimum activity over a broad pH range, between 1.2 and 3.5. At pH values equal to or higher than 3.2, there was no detectable hydrolysis of the protein, even after 120 min of digestion (Fig. 1). The pH in the stomach of infants up to 2 months old is 3.0–4.0 and it can increase after food intake to values above 6.0 (Dupont et al., 2010).

While it is generally recognised that LYS is resistant to pepsin action (Polverino de Laureto et al., 2002), there is not much information on the proteolytic susceptibility of this protein and some discrepancies exist. Thus, Mine et al. (2004) reported its complete hydrolysis after 60 min of treatment at pH 1.0 and an E:S of 1:25 (wt:wt), while Fu, Abbott, and Hatzos (2002) claimed that it resisted more than 60 min at pH 1.2, at an E:S of 13:1 (wt:wt). Ibrahim et al. (2005) found that 40% of the original LYS was hydrolysed after 120 min of digestion at an E:S of 1:50 (wt:wt) and pH 4.0 (conditions that mimicked the infant stomach), giving three peptides with molecular masses of 7365, 5444 and 4317 kDa with a

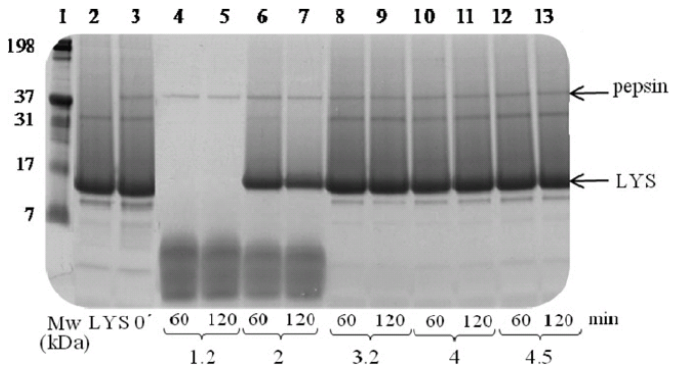

Fig. 1. SDS–PAGE analysis of LYS digested with pepsin, at a pepsin:protein ratio of 1:20 (w:w), at different pHs and hydrolysis times. Lane 1: molecular mass markers; lane 2: LYS; lanes 3, 4 and 5: LYS digested at pH 1.2 for 0, 60 and 120 min; lanes 6 and 7: LYS digested at pH 2.0 for 60 and 120 min; lanes 8 and 9: LYS digested at pH 3.2 for 60 and 120 min; lanes 10 and 11: LYS digested at pH 4.0 for 60 and 120 min; lanes 12 and 13: LYS digested at pH 4.5 for 60 and 120 min.

potent bactericidal activity. According to these authors, this could suggest the important biological role of pepsin hydrolysis of LYS from human milk as a defence system in the stomach of the newborn. However, under our digestion conditions, LYS was completely resistant to pepsin at pH values of 4.0 and 4.5 (Fig. 1).

3.2. Effect of the presence of PC on the gastric digestion of LYS in vitro

Fig. 2 compares the RP-HPLC pattern of *in vitro* gastric digestions performed for 60 min, at pH 2.0, in the absence and presence of PC, a physiological surfactant secreted by the gastric mucosa and also present in the bile. It should be mentioned that, regardless of the addition of PC, there were signs of protein precipitation on adjustment of the gastric digests to pH to 7.0 to irreversibly inactivate pepsin before the RP-HPLC analyses. The precipitate was removed by centrifugation and it was identified by RP-HPLC as unhydrolysed LYS. While residual LYS precipitated from the gastric digests at pH 7.0 solubilisation experiments demonstrated that the intact protein was fully soluble in SGF at different pHs up to 7.7, as well as in 10 mM phosphate buffer at pH 7.0 (results not shown).

As shown in Fig. 2, 6.3 mM PC (an 18:1 M ratio of PC:protein) did not have an important effect on the susceptibility of LYS to hydrolysis by pepsin, although it slightly increased LYS resistance to digestion, decreasing the presence of degradation products, without signs of changes in the fragmentation pattern (Fig. 2b and c). A similar behaviour has been reported for other proteins, such as LA, whose interaction with PC was shown to retard its proteolysis during gastric digestion, a result that was attributed to the partial penetration of LA into PC vesicles (Moreno et al., 2005b). In fact, when LA was digested with pepsin in the absence and presence of PC, the protective effect of PC on LA proteolysis was confirmed (Fig. 3). Thus, in agreement with the results of Moreno et al. (2005b), in the absence of PC, LA was rapidly hydrolysed by pepsin but, when PC was present, there was intact protein remaining after 5 and 15 min. On the other hand, pepsin hydrolysis of other proteins, such as the 2S albumin from Brazil nut (Moreno et al., 2005b, β-Lactoglobulin LG) (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Mandalari, Mackie, Rigby, Wickham, & Mills, 2009), or ovalbumin (OVA) (Martos et al., 2010) is not affected by the presence of PC in SGF at pH 2.5 or 2.0.

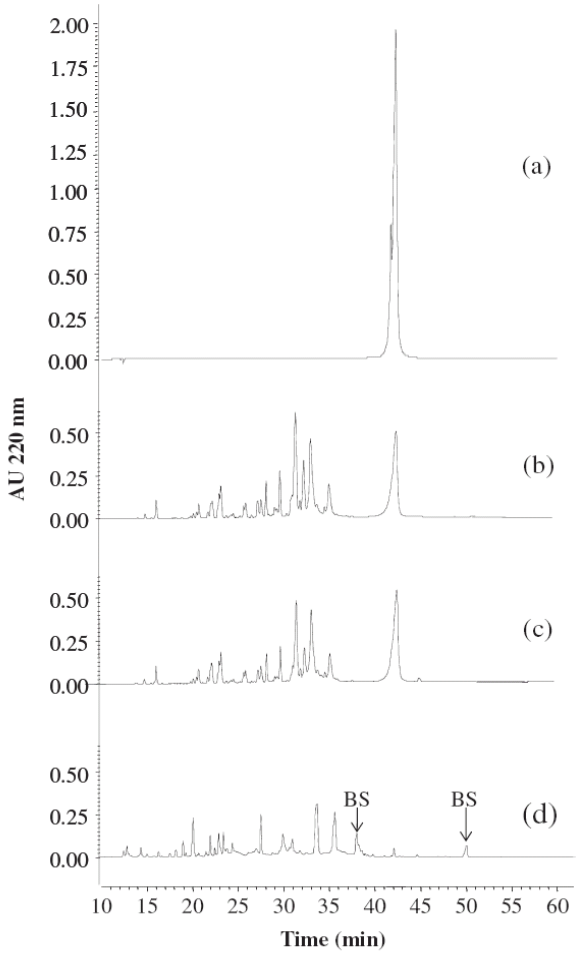

Fig. 2. RP-HPLC patterns of LYS (a) and its digests under different conditions (b–d). *In-vitro* gastric digestions at pH 2.0, for 60 min, in the absence (b); and presence (c) of phosphatidylcholine (PC); and *in-vitro* duodenal digest of (b) for 30 min (d). BS: bile salts.

Hen egg white LYS and bovine LA are homologous to each other as they share similar primary structures, but their unfolding profiles and the stabilities of their native conformations are very different (Cawthern, Permyakov, & Berliner, 1996; Polverino de Laureto et al., 2002). The biological functions of LYS, such as its antimicrobial and immunomodulating properties, have been attributed to its ability to interact with membrane component phospholipids and to penetrate into lipid bilayers, aspects which have been broadly studied (Gorbenko, Loffe, & Kinnunen, 2007; Yuan et al., 2007). Similarly, the interaction of LA with phospholipid membranes and vesicles is of interest because membranes are implicated in the protein folding behaviour and in the ability of LA to regulate lactose synthesis (Cawthern et al., 1996).

The structural features of LYS and LA in the absence and presence of PC were studied by CD and fluorescence. The far-UV CD spectra of LYS (Fig. 4a) showed that the secondary structure of LYS in solution was the same at pH 2.0 and 7.0 and that it was not altered in the presence of PC. These results agree with Witoonsaridsilp, Panyarachun, Sarisuta, and Müller-Goymann (2010), who reported that the secondary structure of LYS entrapped in non-charged and negatively-charged liposomes, with various lipid compositions, does not significantly differ from that in buffer solutions at different pH values. The near-UV CD spectrum of LYS at pH 2.0 was typical of this protein, which does not change with respect to the neutral pH condition (Fig. 4b) (Polverino de Laureto et al., 2002). However, in the presence of PC at pH 7.0, a reduction in the signals in the near-UV region was patent, which suggested a disruption of the tertiary structure.

The far-UV spectrum of LA at neutral pH displayed two minima of ellipticity near 208 and 220 nm (Fig. 4c). Comparatively, at pH 2.0, it showed, approximately, 0.01% losses of alpha helix and beta strand. However, the near-UV spectra showed that the intensity of the dichroic signals in the 250–300 nm region was very low at pH 2.0, in comparison with pH 7.0 (Fig. 4d). This is in agreement with previous reports that indicated that, at acidic pH, LA maintains a native-like content of α-helical structure, but its tertiary structure is almost completely disrupted, due to the formation of a partially folded state named molten globule (Moreno et al., 2005b; Polverino de Laureto et al., 2002). The enhanced flexibility or local unfolding of LA over LYS at acidic pH probably favours cleavage of the former by pepsin, while LYS is highly resistant.

Intrinsic fluorescence emission spectra of LYS at pH 2.0 and 7.0 are shown in Fig. 5a. The presence of six tryptophan residues in LYS, with different spectral contributions because of their different polar environments, results in relatively broad fluorescence spectra (Gorbenko et al., 2007). The lower intensity at acidic pH can be attributed to a quenching effect exerted by protonated acidic groups. Upon titration with PC at pH 2.0, the wavelength of maximum emission (λ_{max}) on excitation at 280 nm changed from 343 nm to 340 nm. Since the transfer of Trp into an environment with a lower polarity usually coincides with a blue shift of the λ_{max}, this suggests that mixing of LYS with PC modified the local

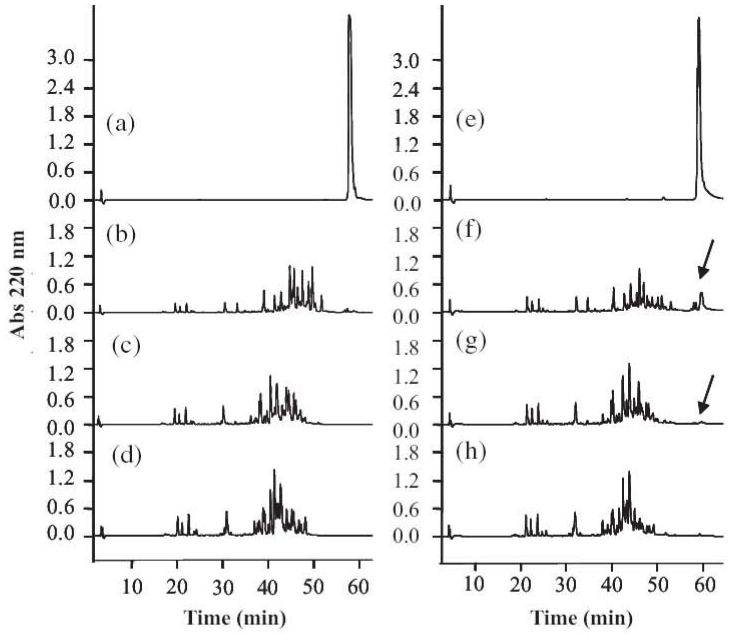

Fig. 3. RP-HPLC patterns of LA (a, e) and its pepsin digests obtained at pH 2.0 in the absence (a–d); and presence (e–h) of phosphatidylcholine (PC); at different hydrolysis times: 0 min (a, e), 5 min (b, f), 15 min (c, g), and 30 min (d, h).

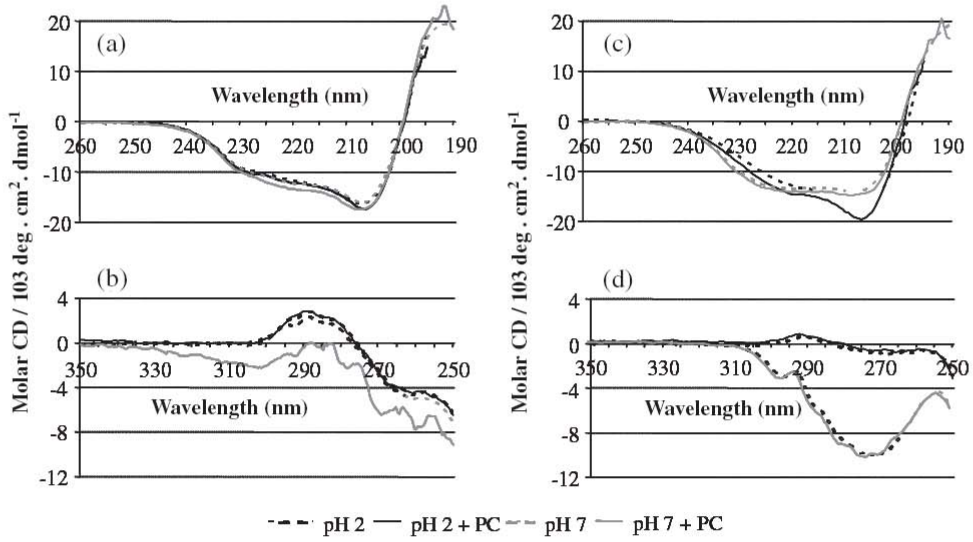

Fig. 4. Circular dichroism spectra in the far (a, c) and near (b, d) UV region of LYS (a, b) and LA (c, d), at pH 2.0 and 7.0 in the absence and presence of phosphatidylcholine (PC).

environment of the protein making it more hydrophobic, possibly through membrane binding. In fact, a blue shift in the spectrum of LYS has been attributed to a shielding effect from water arising from the penetration of the protein into a phospholipid bilayer (Yuan et al., 2007). When different amounts of PC were titrated into a fixed concentration of LYS at acidic pH the fluorescence intensity decreased regularly (Fig. 5c), suggesting an interaction between the phospholipid and the protein that changed the accessibility of Trp residues to the solvent. On the other hand, at neutral pH, fluorescence intensity hardly changed on PC addition.

Regarding bovine LA, the longer wavelength of the λ_{max} and the higher fluorescence intensity of the spectrum at pH 2.0, as compared to pH 7.0, supported that the Trp in the molten globule are more accessible to the solvent than in native LA (Svensson et al., 1999) (Fig. 5b). In this case, titration experiments showed that the fluorescence intensity progressively changed with the concentration of PC, decreasing at pH 2.0 and increasing at pH 7.0 (Fig. 5b and d). This suggests that, at pH 2.0, there is a partial insertion of Trp into the apolar phase of the lipid bilayer, while at pH 7.0, association leads to an increase in Trp accessibility. In fact, at acidic pH, LA behaves as a membrane intrinsic protein and penetrates into PC vesicles. Below its isoelectric point (4.8), hydrophobic forces dominate the interaction of LA and PC, with binding being probably reinforced by the unfolding of the protein that increases surface hydrophobicity (Cawthern et al., 1996; Moreno et al., 2005b). Above its isoelectric point, the interaction with the zwitterionic phospholipid is mainly electrostatic, which induces a loose association with the outer surface of the vesicles (Moreno et al., 2005b). Deep insertion of proteins into membranes has been reported to prevent their degradation on incubation with

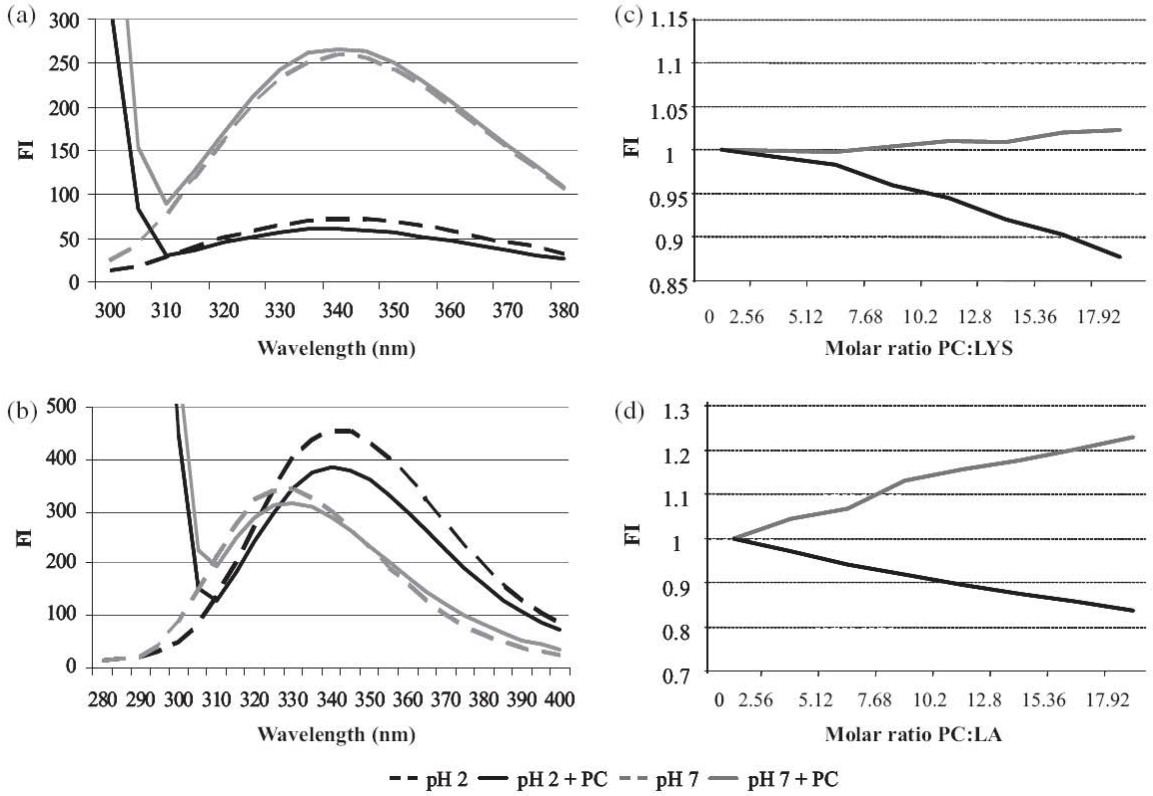

Fig. 5. Fluorescence spectra of LYS (a) and LA (b), at pH 2.0 and 7.0, in the absence and presence of phosphatidylcholine (PC), and corrected LYS (c) and LA (d) titration curves with PC at pH 2.0 and pH 7.0.

proteases, while membrane association, without losing protein integrity, may make them more sensitive to proteolytic degradation (Mogensen et al., 2007).

While LA attains a molten globule estate at pH 2.0, LYS remains native. Nevertheless, LYS could still interact with zwitterionic phospholipids, such as PC, through hydrophobic and polar interactions that could lead to LYS association to PC films. In fact, it has been reported that LYS could be entrapped in non-charged PC liposomes (Witoonsaridsilp et al., 2010). However, penetration is difficult once adsorption has occurred, because LYS is excluded from interacting with the hydrophobic portion of the lipid below the chain transition temperature of PC, which is approximately 41 °C (Mudgil, Torres, & Millar, 2006). On the other hand, LYS, with an isoelectric point near 11, is a highly electropositive protein, that can easily penetrate anionic phospholipid vesicles, such as those formed by phosphatidylserine or phosphatidylglycerol. In this case, LYS binding to anionic vesicles is governed by electrostatic effects that lead to LYS penetration into the lipid phase (Mudgil et al., 2006; Witoonsaridsilp et al., 2010).

3.3. Effect of the presence of BS and PC on the Duodenal digestion of LYS in vitro

The gastric digests of LYS produced after 60 min at pH 2.0, with and without the addition of PC, once adjusted to pH 7.0 and centrifuged, were subjected to an *in vitro* duodenal digestion for 30 min with trypsin and chymotrypsin in the presence of BS. Under those conditions, LYS completely disappeared (Fig. 2d), despite, according to the literature, trypsin and chymotrypsin hardly hydrolysing the protein (During, Porsch, Mahn, Brinkmann, & Gieffers, 1999), or just hydrolysing it partially after overnight incubation at 37 °C (Mine et al., 2004).

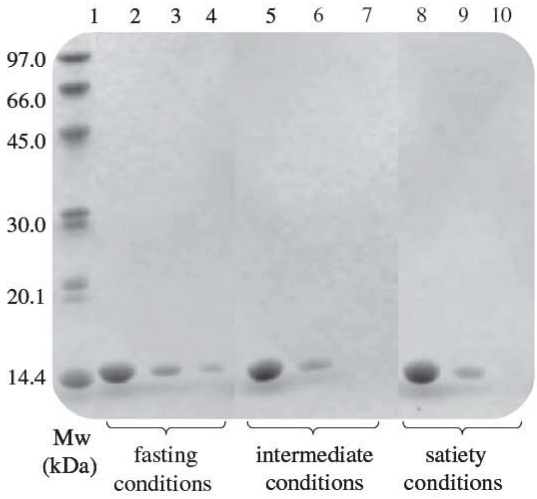

Fig. 6. SDS–PAGE analysis of LYS soluble in 7.6 mM $CaCl_2$ and 20.3 mM Bis–Tris mimicking duodenal conditions typical of a fasted (2, 3, 4); intermediate (5, 6, 7) and fed (8, 9, 10) state. Lane 1: molecular mass markers; lane 2: LYS at pH 7.0; lane 3: LYS at pH 7.0 with 3 mM of each BS and 2.4 mM PC; lane 4: LYS at pH 7.0 with 3 mM of each BS; lane 5: LYS at pH 6.5; lane 6: LYS at pH 6.5 with 6 mM of each BS and 6.5 mM PC; lane 7: LYS at pH 6.5 with 6 mM of each BS; lane 8: LYS at pH 6.0; lane 9: LYS at pH 6.0, with 9 mM of each BS and 7.2 mM PC; lane 10: LYS at pH 6.0 with 9 mM of each BS. BS: bile salts.

To elucidate whether the presence of BS could have an effect on LYS solubility, we incubated the intact protein with three different duodenal digestion media, typical of a fasted state, an intermediate state similar to the duodenal mixture we used for digestion, and a fed state, but without proteases and lipases. Fig. 6 shows the effect

on LYS solubility of the different simulated small intestine conditions.

LYS was fully soluble in 7.6 mM $CaCl_2$ and 20.3 mM Bis–Tris at pH 6.0, 6.5, and 7.0, but it precipitated in the presence of BS. The concentration of amphiphilic bile components in the upper intestine, including BS and PC, increases after a meal, while the pH decreases (Kostewicz et al., 2002). As shown in Fig. 6, the higher the bile salt concentration the lower the amount of soluble LYS. This indicated that the disappearance of the protein could not be totally attributed to its hydrolysis by duodenal enzymes but, to some extent, to its precipitation with BS in the duodenal medium. It should be noted that the presence of PC partially avoided LYS precipitation, suggesting a positive effect on solubilisation of the mixed bile salt-PC micelles present in the duodenal medium (Mandalari et al., 2009). It should be noted that the BS concentration used was always over the critical micelle concentration, 3.5 mM (Kaukonen et al., 2004).

Although, according to Burnett et al. (2002), BS exert a solubilising effect, removing proteins adsorbed to emulsions and favouring their resolubilisation in aqueous phases, our results show that it is likely that LYS precipitates in the duodenum at pH values, BS and PC concentrations representative of a fed state and, to a lesser extent, of a fasted state. In fact, when LYS is administered orally to human patients (as used for the treatment of chronic sinusitis and to promote expectoration in the case of respiratory disease), food intake negatively affects the uptake of the enzyme, with the maximum levels detected being almost 10-fold lower than those after an identical dose taken after an overnight fast (Hashida, Ishikawa, Nakamichi, & Sekino, 2002).

Insolubilisation of LYS in the presence of BS could impair its hydrolysis by pancreatic enzymes, affecting its presence in the intestinal tract. Despite its molecular weight, LYS can be effectively absorbed and its uptake occurs preferentially in the upper intestine (Takano, Koyama, Nishikawa, Murakami, & Yumoto, 2004). Absorption of the intact protein could be particularly relevant in infants, whose immature gastrointestinal epithelium allows more proteins to cross the barrier into the circulatory system. Furthermore, while LYS precipitation could play a role in the uptake of LYS by intestinal brush border membranes and its presentation to the immune system, it may positively enhance the proportion of antimicrobial agents that can play a defensive role in lower parts of the intestine.

3.4. Effect of digestion on IgG and IgE binding

The IgG and IgE-binding properties of the gastric digests obtained after 60 min were examined by inhibition ELISA. The IgG-binding was estimated with a polyclonal commercial antibody against LYS raised in rabbit, while for IgE-binding we used sera of patients allergic to egg with proved immunoreactivity against LYS.

The digests exhibited a substantial residual antigenicity both against IgG and IgE (Fig. 7a and b), which could only be somewhat attributed to the presence of residual LYS, since, as already mentioned, LYS partially precipitated (around 40–45% as estimated by RP-HPLC) when the pH was adjusted to 7.0 to inactivate pepsin. This indicated that some of the proteolysis products retained a noticeable reactivity against IgG and IgE and that, in fact, the reactivity of the digests was comparatively higher than that of the intact protein. The highest IgG-binding was detected in the gastric digests obtained in the presence of PC ($p < 0.05$), which also contained more intact protein than those produced in the absence of PC as showed above (Fig. 3). Regarding IgE-binding, as expected, the responses were shown to vary depending on the patient's individual susceptibility. In this case, there were no differences between the hydrolysates obtained with or without PC. Overall, these results show that *in vitro* gastric digestion of LYS led to the formation of peptides which carried an important epitope load and thus, the potential to be allergenic.

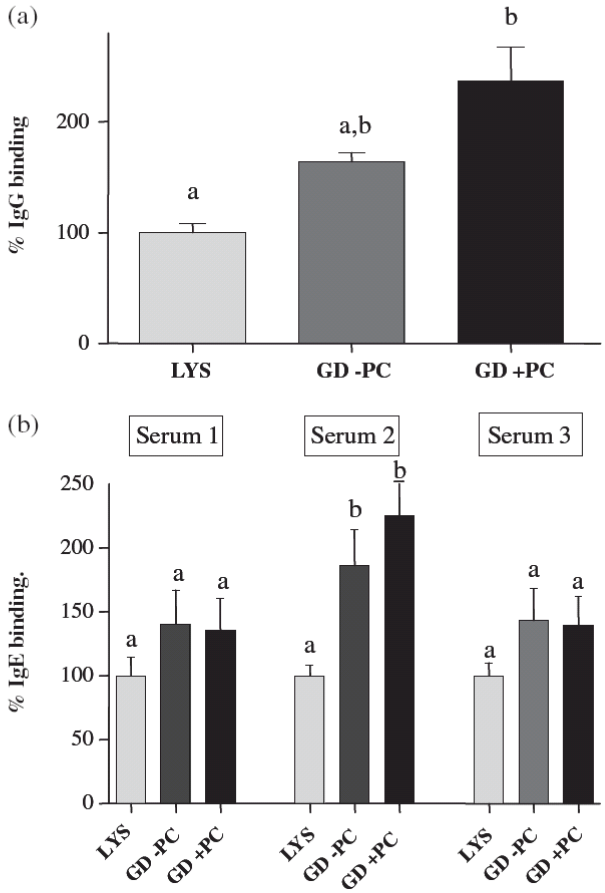

Fig. 7. Inhibition ELISA response against rabbit polyclonal anti-LYS IgG (a) and human IgE (b) of LYS and its pepsin digests obtained at pH 2.0 in the absence (−PC) and presence of phosphatidylcholine (+PC) for 60 min. Human sera were IgE >100 kU/L. The IgG- and IgE-binding capacities of the digests were expressed as percentages of the IC_{50} of the intact protein. Significant differences ($p < 0.05$) were evaluated by one-way analysis of variance. Different letters above the bars indicate significant differences ($p < 0.05$). Error bars correspond to the mean ± standard error ($n = 3$).

4. Conclusions

Digestion of LYS with pepsin was conducted at an enzyme: substrate ratio of 1:20 (wt/wt) (172 U/mg), considered as a physiological ratio, under different pHs that simulated various conditions in the stomach. Hydrolysis of the protein only occurred at pH 1.2 and 2.0, typical of a fasted state, with the formation of degradation products of less than 4–5 kDa that resisted pepsin action for at least 2 h of digestion. At pH values equal or higher than 3.2, typical of a fed state or of immature stomach functions, there was no detectable hydrolysis of the protein, even after 120 min of digestion. The presence of PC, a physiological surfactant, slightly increased LYS resistance to digestion, decreasing the presence of degradation products, and a similar behaviour was found for its homologous protein LA. At acidic pH, the enhanced flexibility or local unfolding of LA over LYS, as determined by circular dichroism spectroscopy probably favoured the cleavage of the former by pepsin, while LYS was more resistant. Intrinsic fluorescence emission spectra of LYS and LA at pH 2.0, on addition of PC, suggested that the local environment of the proteins became more hydrophobic, possibly through membrane binding. It is, therefore, likely that,

at acidic pH, both proteins interact with PC, leading to their association with PC films.

IgE-binding experiments using sera of patients allergic to egg showed that *in vitro* gastric digestion of LYS led to the formation of peptides which carried an important epitope load and could have the potential to be allergenic. The identification of these peptides and their resistance to hydrolysis by pancreatic proteinases are currently being addressed. On the other hand, our results show that unhydrolysed LYS precipitated at pH values, and BS and PC concentrations representative of a duodenal fed state (characterised by high BS and PC concentrations and low pH) and, to a lesser extent, of a fasted state. LYS precipitation in the presence of BS could impair its hydrolysis by pancreatic enzymes, either affecting the amount of immunoreactive protein that is absorbed or the proportion of antimicrobial agents that can play a defensive role in lower parts of the intestine.

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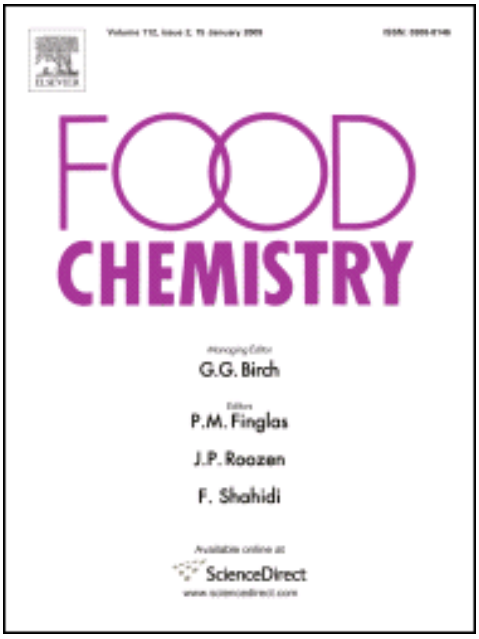

Food. Chem. (Enviado)

Human IgE binding and *in vitro* digestion of S-OVA

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Food Chemistry

Human IgE binding and *in vitro* digestion of S-OVA

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Abstract

S-OVA, a more thermostable form of ovalbumin (OVA), was formed from native OVA or egg white *in vitro*, by heating at high pH, and by storage at low temperatures. S-OVA showed a much lower reactivity against IgE than OVA, although this difference in IgE binding was minimized after simulated gastro intestinal digestion, despite S-OVA was more resistant to proteolysis, particularly to pepsin, than its native form. It is, therefore, likely that the spontaneous transformation of OVA to S-OVA does not affect its ability to sensitize or trigger allergic reactions at the duodenal level. These results are discussed in the light of the described conformational changes reported to occur in the transition between OVA and S-OVA.

Abbreviations:

DD: duodenal digestion; EW: egg white; GD: gastric digestion; OVA: ovalbumin; SGF: simulated gastric fluid; S-OVA: S-ovalbumin.

Keywords:

S-ovalbumin; Ovalbumin; *in vitro* gastro intestinal digestion; IgE-binding;

1. Introduction

Ovalbumin (OVA or gal d 2) is the major egg allergen making up to 54% of the weight of egg white (EW) proteins (Lechevalier, Croguennec, Nau & Guérin-Dubiard, 2007). It is a phosphoglycoprotein with a molecular mass of 45 kDa (Nisbet, Saundry, Moir, Fothergill & Fothergill, 1981), comprised of 385 amino acids (Mcreynolds et al., 1978) and classified as a member of the serine proteinase inhibitor family, although it has no inhibitory activity in its native form (Hunt & Dayhoff, 1980; Stein, Finch, Turnell, Mclaughlin & Carrell, 1990).

During the storage of eggs, OVA is converted into a more heat-stable protein, S-ovalbumin (S-OVA) (Smith, 1964; Smith & Back, 1965), with an increased denaturation temperature of 92.5ºC as measured by differential scanning calorimetry (DSC) (Donovan, Mapes, Davis & Garibaldi, 1975). The transition into an intermediate form between OVA and S-OVA, which has been described in some studies, seems to be necessary for S-OVA formation (Donovan, et al., 1975; Hegg, Martens & Lofqvist, 1979; Ishimaru, Ito, Tanaka & Matsudomi, 2010). The conformational features of S-OVA are still under research but, so far, it is accepted that the overall structure of S-OVA is quite similar to that of native OVA, except that the amino acids Ser164, Ser236, and Ser320 take the D-configuration and the side chains of Phe99 and Met241 undergo conformational changes (Yamasaki, Takahashi & Hirose, 2003; Ishimaru et al., 2010). An increase of anti parallel β-sheet conformation in detriment of α-helix has also been reported (Kint & Tomimatsu, 1979).

S-OVA represents up to 5% of the content of native OVA in fresh EW, but more than half of OVA is converted to S-OVA by the time the eggs reach the grocery store and, finally, the consumer (Lechevalier et al., 2007). After 6 months of storage at low temperature, the percentage of S-OVA can reach 81% (Vadehra & Nath, 1973). S-

OVA can also be formed *in vitro* at alkaline pH and high temperature, for example by incubation at 50ºC for 20 h in 100 mM sodium phosphate at pH 10 (Donovan et al., 1975). The presence of S-OVA in eggs is usually related to a loss of EW functionality, because it induces runny whites with poor congealing capacity (Shitamori, Kojima & Nakamura, 1984). Most of the work on S-OVA has focused on the quality of stored eggs and related products (Huntington & Stein, 2001), but not on other important topics, such as its proteinase inhibitory activity, its allergenicity or digestibility (Smith & Back, 1968).

Resistance to digestion is considered one of the main features that reflect a food allergen's capacity to stimulate a specific immune response (Astwood, Leach & Fuchs, 1996). The higher structural stability reported for S-OVA may contribute to keep its integrity through the gastro duodenal tract, increasing its ability to sensitize or trigger allergic responses. From this background, the aim of this work was to assess how the transformation of OVA into S-OVA (either by storage or *in vitro*) affects its digestibility, estimated by using an *in vitro* model that mimics physiological conditions, as well as its allergenicity, measured in terms of human-IgE binding by inhibition ELISA.

2. Material and Methods

2.1 Formation of S-OVA *in vitro*

S-OVA samples were prepared from a 50 g/L solution of hen´s egg OVA grade VI from Sigma-Aldrich (St. Louis, MO, USA) in milli-Q water, following Smith et al. (1965) and from EW, obtained from fresh eggs, following Castellano, Barteri, Bianconi, Bruni, DellaLong & Paolinelli (1996). The pH of the samples was set at 9.9 by drop wise addition of 0.5 M sodium hydroxide and they were heat treated in a water bath at

55ºC for 16 h in 50 mL sealed centrifuge tubes. Then, the samples were cooled down to room temperature (RT) and the pH was adjusted to 4.7 with 0.1 M acetic acid. Denatured proteins were removed by centrifugation at 14,700 g for 10 min and the supernatant was dialysed extensively overnight at 4ºC by using a Spectra/por® Membrane (MWCO 6-8000, Spectra Medical Industries, Inc., Rohini, New Delhi, India). The samples were freeze dried and kept at -20ºC until use. All experiments were carried out in duplicate.

2.2 Formation of S-OVA on storage

Fresh eggs were stored at 4ºC for 6 months. The EW, manually separated from the yolks, were pooled and gently homogenized to avoid foam formation. The pH and weight were checked and the samples were freeze-dried and kept at –20ºC until further analyses. All experiments were carried out in duplicate.

2.3 Differential scanning calorimetry (DSC)

Freeze dried samples were dissolved in 0.05 M Tris-buffer at pH 9.01 to a final concentration of 40 g/L. The denaturation temperature of the samples was determined by DSC (Q1000, TA Instruments Inc., New Castle, DE, USA) by heating from 20 to 120ºC in hermetic aluminium crucibles at a heating rate of 10ºC/min. The denaturation temperatures were determined as the midpoint temperature of the endothermic peak using the Universal Analysis 2000 software (TA Instruments Inc.).

2.4 In vitro gastro duodenal digestion.

In vitro digestions were carried out in duplicate as previously described (Moreno, Mellon, Wickham, Bottrill & Mills, 2005; Martos, Contreras, Molina & López-Fandiño, 2010). Freeze-dried samples were dissolved in simulated gastric fluid (SGF, 35 mM NaCl pH 2), preheated for 15 min at 37 ºC and porcine pepsin (EC 3.4.23.1, 3440 units/mg, Sigma-Aldrich) was added at an enzyme/substrate ratio of 1:20

w/w. Aliquots of the gastric digestions were taken at 30 and 60 min and the reaction stopped by raising the pH to 7.0 with 1M sodium bicarbonate, resulting in a final protein concentration of 5.07 mg/mL..

The simulated duodenal digestion was performed on the 60 min gastric digests at pH 7.0. 1 M calcium cloride, 0.25 M bis-Tris, pH 6.5, and a 0.125 M bile salt mixture, containing equimolar quantities of sodium taurocholate (Sigma-Aldrich) and glycodeoxycholic acid (Sigma-Aldrich), were added. The mixture was heated at 37 ºC for 15 min, and the following enzymes dissolved in milli-Q water were added in this order: Corolase PP (AB Enzymes GmbH, Darmstadt, Germany), porcine pancreatic lipase (EC 232-619-9, Sigma-Aldrich), and colipase (EC 259-490-1, Sigma-Aldrich). Aliquots were taken at 30 and 60 min of duodenal digestion and the enzyme activity was stopped by heating at 80ºC for 5 min. The final composition of the mixture was 4.12 mg/mL protein, 6.15 mM of each bile salt, 20.3 mM bis-Tris, 7.6 mM calcium chloride, 28.9 units of lipase/mg and Colipase and Corolase PP enzyme/substrate ratios were 1:895 w/w and 1:25 w/w, respectively. All the aliquots were kept at -20ºC until further use.

2.5 Reverse phase – high performance liquid chromatography (RP-HPLC).

Aliquots taken during gastro duodenal digestion were dissolved in milli-Q water to a final concentration of 2.5 mg/mL. After filtration through 0.2 μm nitrocellulose filters, the digests were separated by RP-HPLC using a Hi-Pore RP-318 (250 x 4.6 mm) column (Bio-Rad) in a Waters 600 HPLC (Waters Corporation, Milford, MA, USA) equipped with a 717 plus autosampler and a 2487 dual wavelength absorbance detector. Elution was performed using 0.37% (v/v) trifluoroacetic acid in milli-Q water, as solvent A, and 0.27% (v/v) trifluoroacetic acid in acetonitrile, as solvent B. Elution was performed as described by Quirós, Chicón, Recio & López-Fandiño (2007) with slight

modifications. In brief, a linear gradient of 0-60% B was applied over 60 min. After 35 min at 60% B, 100% B was reached in 1 min, maintained during 10 min, and finally decreased to 0% in 1 min. The flow rate was maintained at 1 ml/ min and peptides were detected by monitoring at 220 nm. Data were processed by using Empower 2 Software (Waters Corporation).

2.6 Human-IgE Binding by Inhibition ELISA.

Individual serum samples from children with proven allergy to EW were collected at the Hospital General Universitario Gregorio Marañón. All patients showed specific IgE antibodies towards EW and OVA (**Table 1**), as estimated by the Immuno-CAP method (Pharmacia Diagnostic, Uppsala, Sweden) and exhibited clinical allergic symptoms. Inhibition ELISA followed Jiménez-Saiz, Martos, Carrillo, López-Fandiño & Molina (2011) with slight variations: commercial OVA diluted in 0.01 M phosphate buffer, pH 7.4 (PBS) to 10 µg/mL was used as a coating a coating agent for the analyses of OVA and *in vitro*-formed S-OVA (from OVA or EW), while EW was used as coating agent for the analyses of EW and stored EW; polyclonal rabbit anti-human IgE (A0094, Dako, Glostrup, Denmark) and polyclonal swine anti-rabbit immunoglobulins labeled with horseradish peroxidase (P0399, Dako) were used diluted 1:1000 and 1:2000 (v/v), respectively, in PBS containing 0.05% Tween 20 (PBST). A nonlinear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoidal curve of inhibition dose-response with variable slope, from which the IC50 (the concentration that binds 50% of seric IgE) was obtained with the program GraphPad Prism® package (GraphPad Software Inc., San Diego, CA, USA). The IgE binding capacity was expressed as the percentage of the IC50 of the intact protein: OVA for the analyses of OVA and *in vitro*-formed S-OVA (from OVA or EW) and EW for the analyses of EW and stored EW.

3. Results and Discussion

3.1 S-OVA formation

S-OVA formation was confirmed by DSC analyses (**Figure 1)**. Native OVA showed a denaturation temperature (T_D) of 82ºC, in agreement with previous studies (Hammershoj, Larsen, Andersen & Qvist, 2002), and its i*n vitro* incubation under alkaline conditions produced a clear shift in the endothermic peak and a T_D of 90.3ºC, close to the value of 92ºC reported by Donovan et al. (1975) for S-OVA. Similarly, when fresh EW was used as starting material for the i*n vitro* conversion, formation of S-OVA was characterized by a marked displacement of the endothermic peak and an increase in T_D from 81.7ºC to 91.5ºC. The attempts to prepare S-OVA from fresh EW upon storage led to smaller increase in T_D, which was attributed to the low pH attained after the 6-month storage period (9.07). The DSC thermograms suggested that storage at 4ºC for 6 months probably yielded a mixture of OVA, intermediate S-OVA and S-OVA (**Figure 1**).

3.2 In vitro digestion

OVA and S-OVA were digested by using an experimental model in two steps, which mimics gastric and duodenal physiological conditions (Moreno et al., 2005; Martos et al., 2010). OVA and S-OVA formed from commercial OVA or EW by *in vitro* treatments were indistinguishable by RP-HPLC (**Figure 2**) and, in all cases, a large peak of intact protein was still present after pepsin digestion that partially resisted pancreatic enzymes. The low susceptibility of OVA to proteolysis is in agreement with previous studies (Takagi, Teshima, Okunuki & Sawada, 2003; Martos et al., 2010). Moreover, S-OVA formed *in vitro* was even more resistant than OVA to the hydrolysis by pepsin, as revealed by a higher amount of intact protein and a lower presence of degradation products after 30 and 60 min of simulated gastric digestion. The enhanced

susceptibility to proteolysis of S-OVA with respect to OVA was not so evident after the simulated duodenal digestions. EW stored for 6 months was also more prone to pepsin action when compared with fresh EW (**Figure 3**), except that the effect was less pronounced, probably because of the presence of both OVA and S-OVA in the stored sample, as explained above.

From these results, it can be drawn that S-OVA is more resistant to *in vitro* digestion under physiological conditions, possibly as a result of the structural changes associated to the transition of OVA into S-OVA that provide S-OVA with grater stability, compactness and hydrophobicity (Huntington et al., 2001).

3.3 Human-IgE binding

The IgE-binding capacity of OVA, S-OVA and their duodenal digests were compared by competitive ELISA using human sera from egg allergic patients (**Figure 4**). Even if the individual sera used varied in their specificity towards the egg proteins and their digests, overall, S-OVA formed *in vitro* showed a much lower reactivity against IgE than native OVA (Fig 4A and B). This lower IgE-binding could be explained because of the structural changes undergone by S-OVA, in particular, those involving the amino acids Phe99, Ser164 and Ser320, all of which form part of previously reported IgE epitopes recognized by allergic subjects, such as the large CNBr-fragments 41-172 and 301-385 that, according to Kahlert, Petersen, Becker & Schlaak (1992), react with patients' IgE. Mine and Rupa (2003) determined the entire mapping of the IgE-binding epitopes in the primary sequence of OVA, finding five distinct regions mainly composed of hydrophobic residues, one of which (95-102) included Phe99. Furthermore, according to Yamasaki et al. (2003), the conformational change in Phe99 side chain in S-OVA induces a decrease in the solvent accessibility of the surrounding residues which include Phe378, a residue that forms part of an

allergenic epitope of OVA which is very accessibly located at the surface of the protein (residues 347-385, Honma et al., 1996). On the other hand, the observed 2-5% loss of α-helix and the 3-4% increase in the β-sheet content in S-OVA, as well as its higher binding capacity against a hydrophobic fluorescent dye, are assumed to result from a partial denaturation during the alkaline treatment (Yamasaki et al., 2003) that could have contributed to the rupture of conformational epitopes with the subsequent reduction in IgE-binding. Regarding EW stored at 4ºC, competitive ELISA showed that it exhibited an IgE binding closer to than that of native EW, with some differences depending on the individual serum used (Fig 4C). This could be attributed to the observation that native OVA was only partially converted into S-OVA and to the presence of other immunogenic egg-white proteins in the stored EW (such as ovomucoid and lysozyme) that could also contribute to its IgE-binding.

Despite the lower IgE-binding of S-OVA and its higher stability towards digestion, the duodenal digests of OVA and S-OVA formed *in vitro* showed a reduced but comparable reactivity towards IgE, and the same happened in the case of fresh and stored EW. This could be explained by the similarity in their duodenal digestion patterns (**Figures 2 and 3**) that point to a similar contribution of peptides containing intact binding sites recognizable by serum IgE, which are the compounds most probably responsible for the low, but detectable ELISA response. It is therefore, likely that the transformation of OVA to S-OVA does not affect its ability to sensitize or trigger allergic reactions at the duodenal level.

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The authors have declared no conflict of financial/commercial interest

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Figure captions.

Figure 1. DSC thermograms with endothermic heat flow curves of commercial OVA (a), fresh egg white (EW) (c), S-OVA formed *in vitro* from commercial OVA (b), S-OVA formed *in vitro* from fresh EW (d), EW after and 6 months of storage at 4ºC (e).

Figure 2. RP-HPLC patterns corresponding to native OVA (A) and S-OVA subjected to simulated gastric (DG) and duodenal (DD) digestions for 30 and 60 min. S-OVA was formed *in vitro* by heating to 55ºC at high pH either from commercial OVA (B) or from fresh egg white (C).

Figure 3. RP-HPLC patterns corresponding to fresh egg white (EW) (A), and EW stored for 6 months at 4ºC (B), subjected to simulated gastric (DG) and duodenal (DD) digestions for 30 and 60 min. LYS: lysozyme; OM: ovomucoid.

Figure 4. Inhibition ELISA response against human IgE of S-OVA formed *in vitro* from commercial OVA (a), from egg white (EW) (b) and formed upon storage for 6 months at 4ºC from EW (c). The IgE-binding capacities were expressed as the percentage of the IC50 of the intact protein: OVA for the analyses of OVA and *in vitro*-formed S-OVA (from OVA or EW) (A and B) and EW for the analyses of EW and stored EW (C). Bars correspond to the 95% confidence intervals.

Tables

Table 1. Specific IgE levels (kU/L) towards egg, egg white (EW), ovalbumin (OVA) and ovomucoid (OM) of the sera used in the study.

Patient	IgE levels (kU/L)		
	Egg white	OVA	OM
1	-	62	80
2	100	100	97.1
3	Egg > 100		
4	>100	78.9	69.2
5	Egg > 100		

Figures

Figure 1

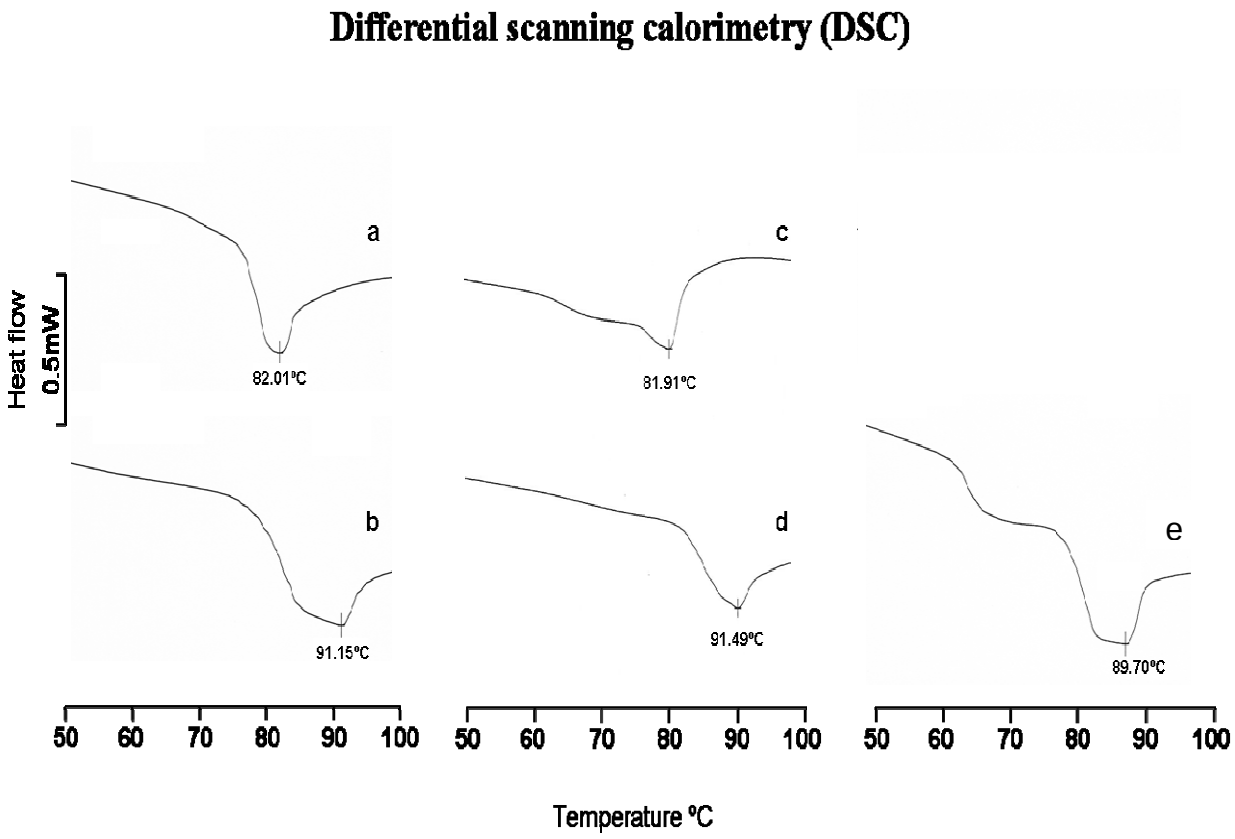


Figure 2

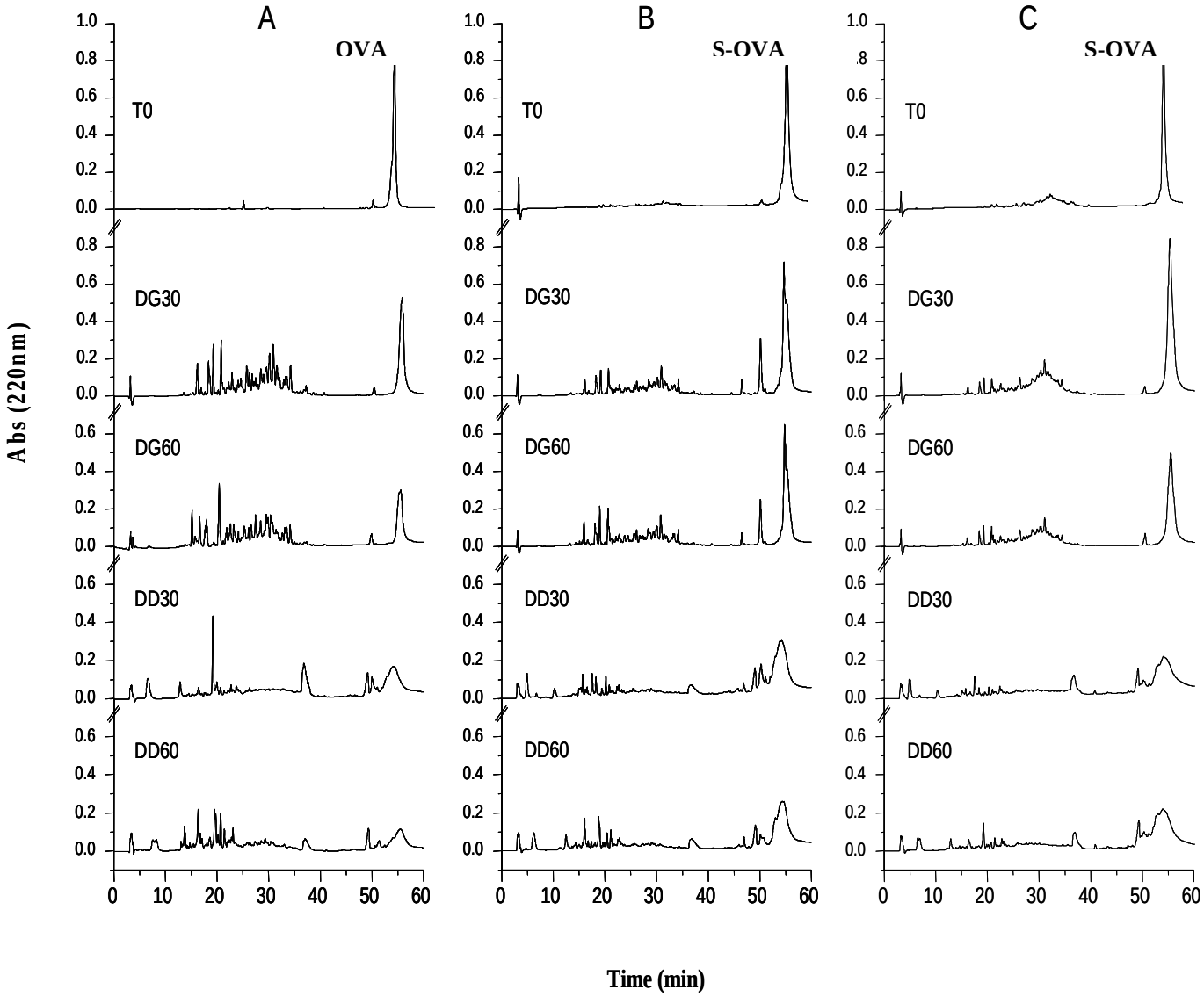
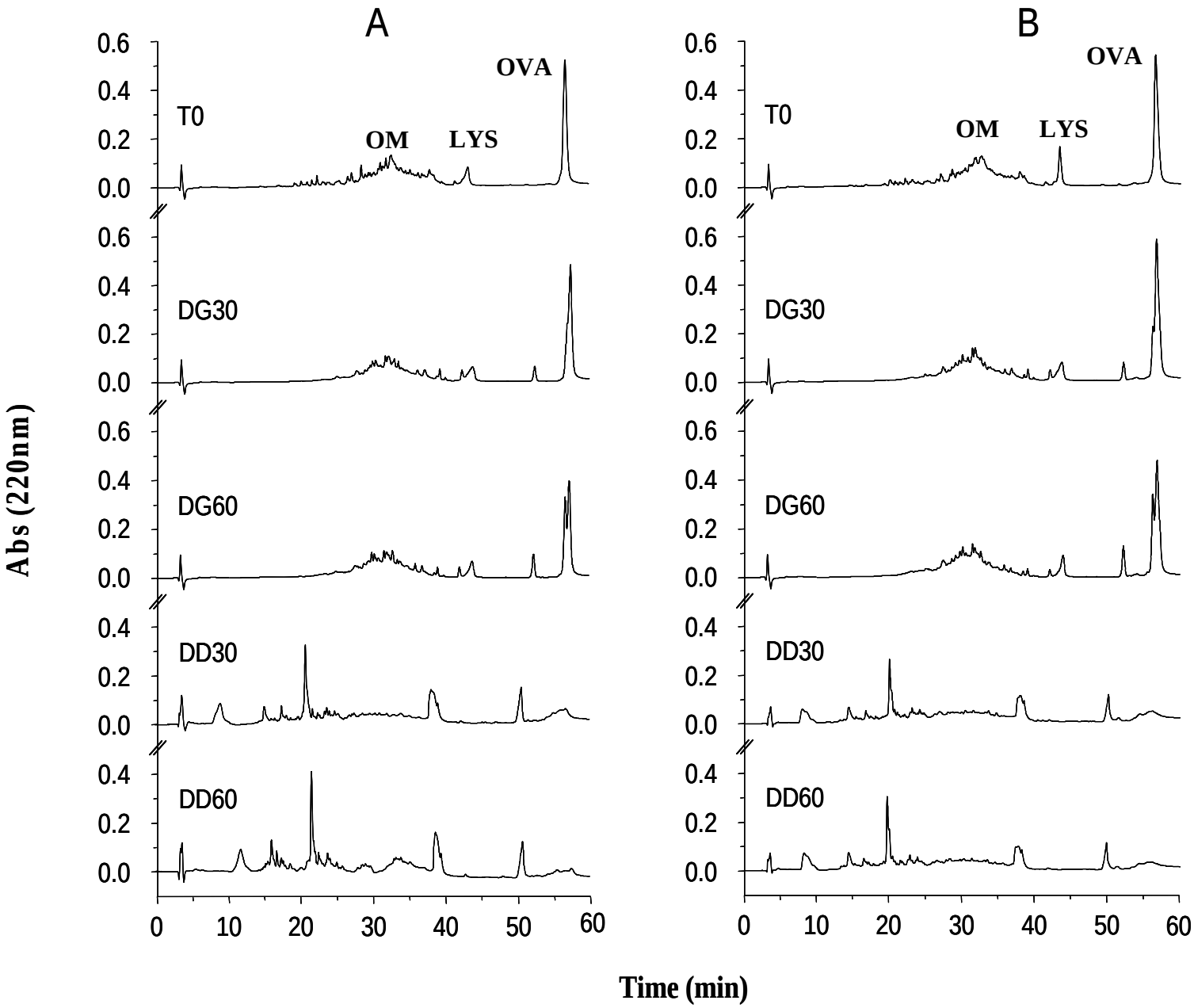


Figure 3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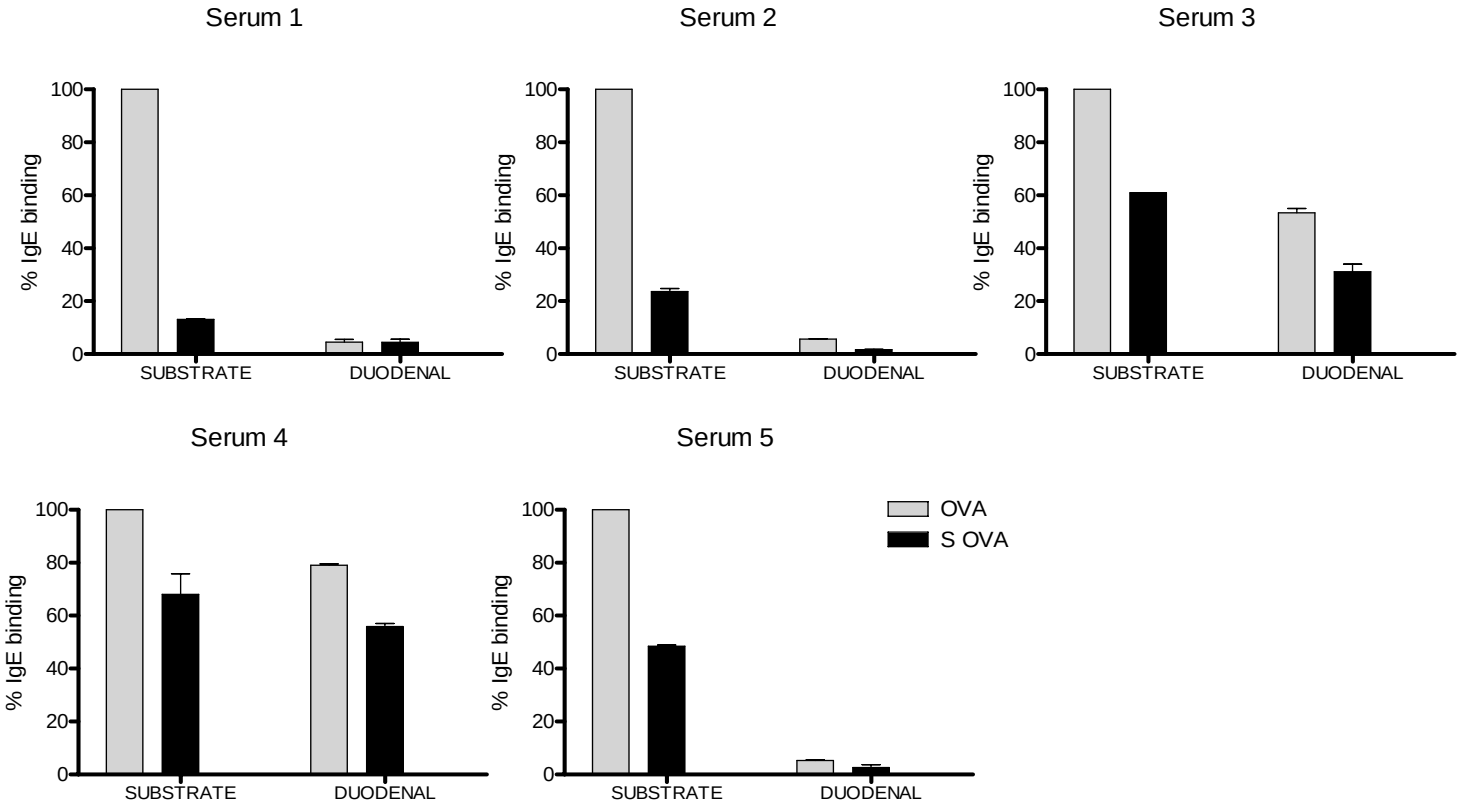
Abs (220nm)
Time (min)

Figure 4

4A



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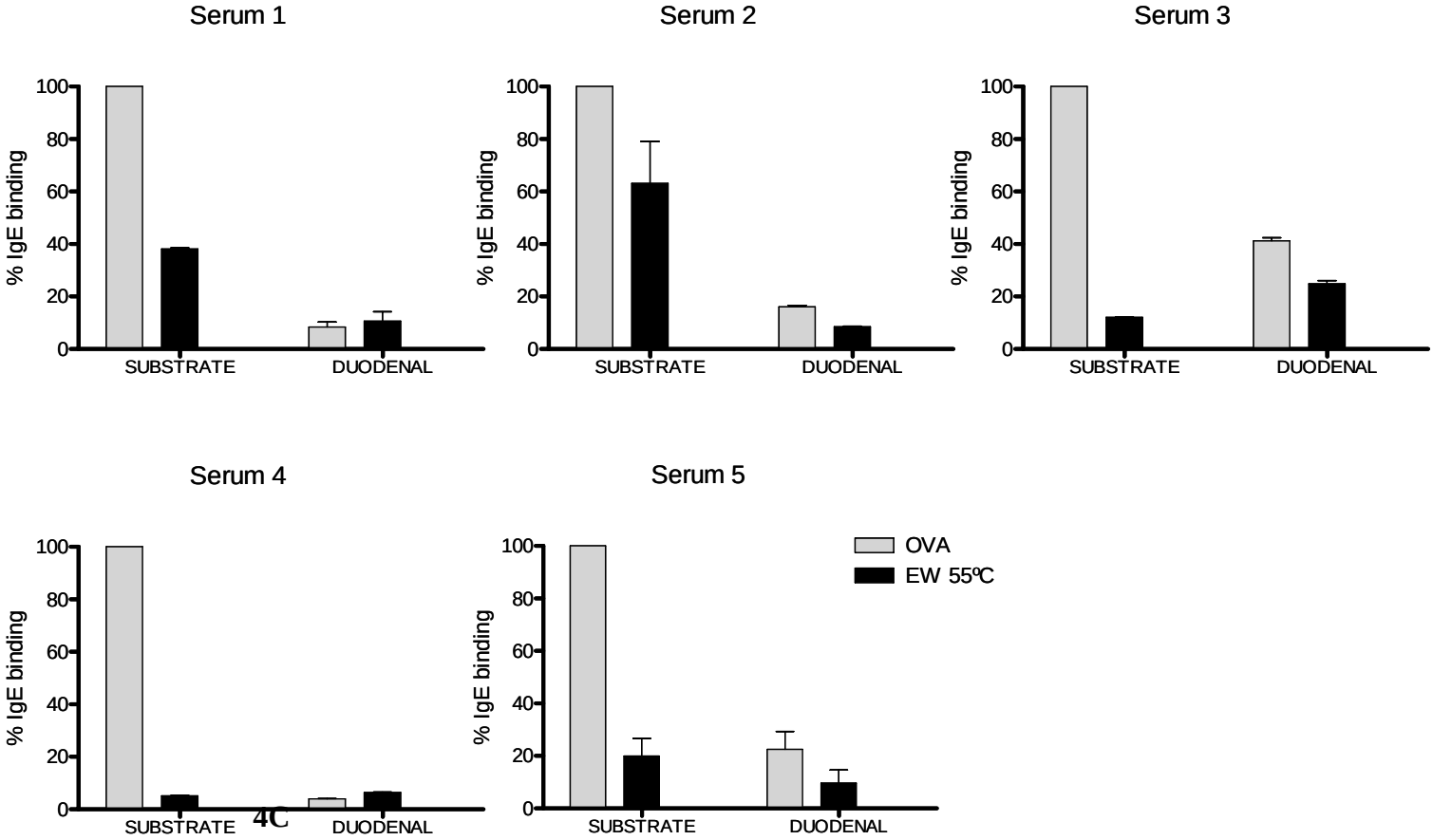

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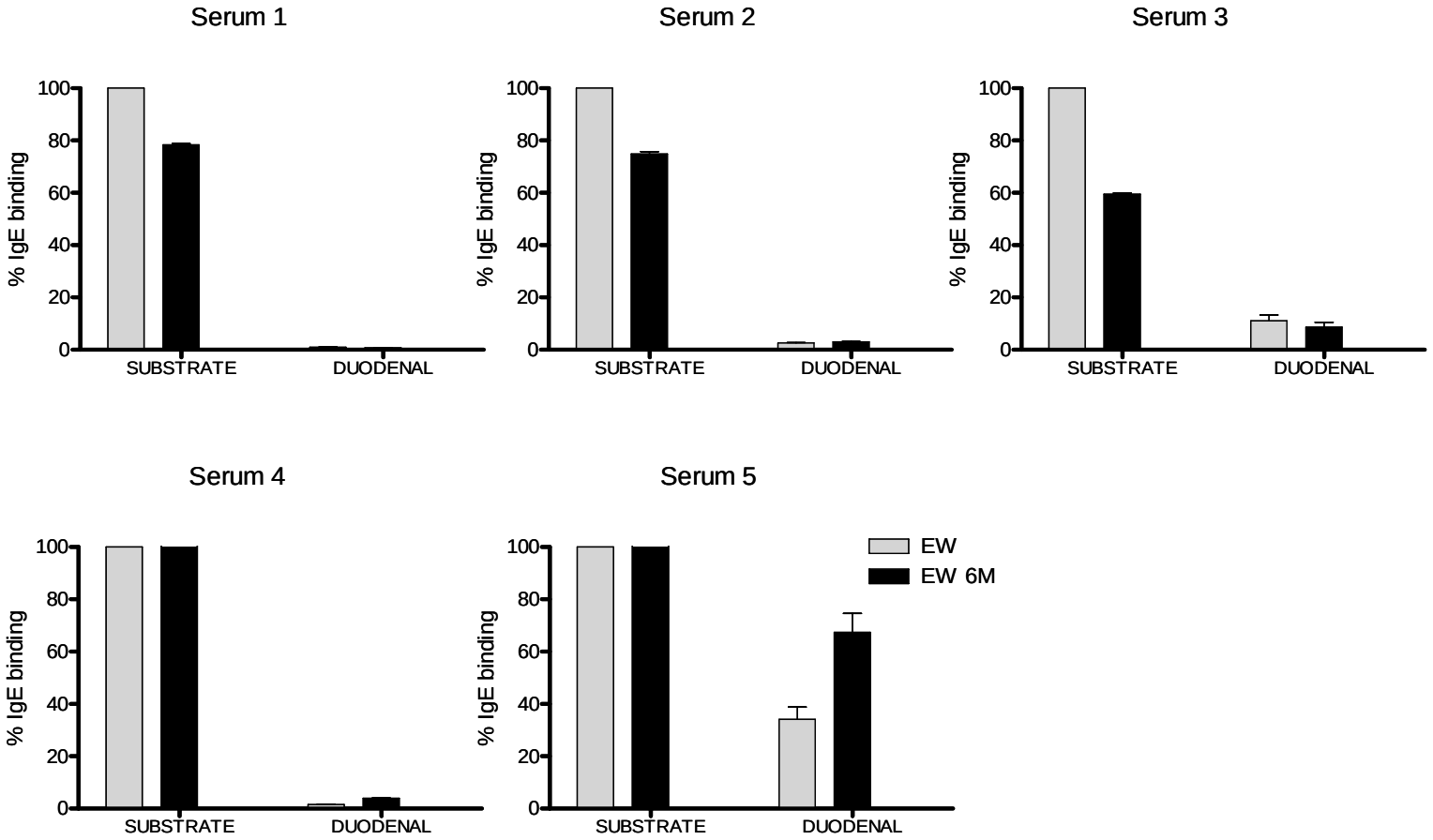
Serum 1
Serum 2
Serum 3
Serum 4
Serum 5
% IgE binding
100
80
60
40
20
0
SUBSTRATE
DUODENAL
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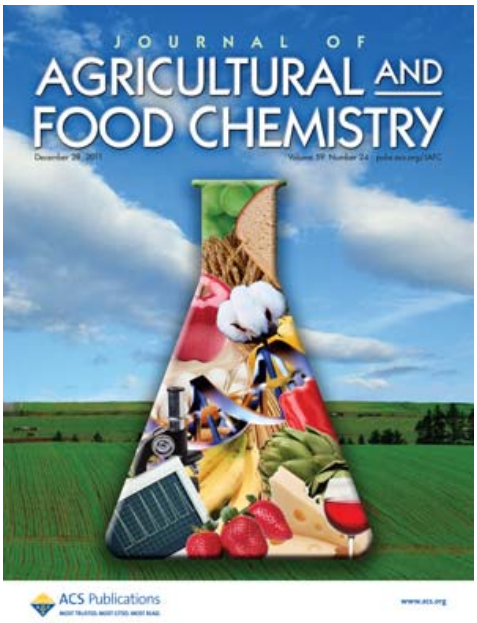

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Human Immunoglobulin E (IgE) Binding to Heated and Glycated Ovalbumin and Ovomucoid before and after in Vitro Digestion

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ABSTRACT: This study focuses on the effect of heating and Maillard reaction (MR) on the in vitro digestibility and rabbit IgG- and human IgE-binding properties of ovalbumin (OVA) and ovomucoid (OM) to estimate the impact of processing on their allergenicity. With the human sera studied, heat treatment significantly reduced IgE binding to both OVA and OM, whereas MR reduced the IgE binding to OVA but increased IgE binding to OM. In contrast, heat treatment significantly favored OVA digestibility but glycation impaired it, and these treatments did not affect the digestibility of OM. The changes observed in the digestibility affected the immunogenicity of the digests accordingly, so that the higher the digestibility, the lower the antibody binding. Heat treatment and glycation by MR showed an influence on the potential allergenicity of the main egg white proteins that could be related to their resistance to denaturation and digestive enzymes.

KEYWORDS: glycation, heating, IgE binding, IgG binding, in vitro digestion, ovalbumin, ovomucoid

INTRODUCTION

Food-induced allergies are recognized as a worldwide health problem. Among them, egg allergy is one of the most frequent hypersensitivities in childhood,[1] with an estimated prevalence that varies between 0.5 and 2%.[2] Nowadays, dietary avoidance of eggs and egg-containing foods is the main approach for treating egg-allergic patients, although it is a difficult task because of the ubiquitous presence of egg proteins in many food products due to their unique functional properties, such as foaming, emulsifying, and gelling.[3]

The major hen egg allergens, ovalbumin (OVA) and ovomucoid (OM), are present in the egg white, OVA (*Gal d 2*, 45 kDa) being the most abundant protein in the egg white (54%, w/w) and OM (*Gal d 1*, 28 kDa, 11%, w/w) the immunodominant protein.[4] Their allergenic potential might be affected by processing, which can hide, destroy, or disclose allergenic epitopes through conformational changes.[5,6] Such modifications may also change protein digestibility and, consequently, the ability to sensitize and elicit the immune response.[7]

Physicochemical changes caused by heat treatment on pure proteins are often associated either with a decrease in allergenicity or with no significant effect, depending on the heat stability of the proteins and their susceptibility to unfold and lose conformational epitopes. For instance, in the case of the milk whey protein β-lactoglobulin, heat-induced denaturation is not sufficient to abolish its allergenicity, but it increases its digestibility, decreasing the ability of the protein to elicit an allergic response.[8] In addition, the effect of the food matrix during processing cannot be ignored. There is evidence that heating of proteins in the presence of sugars, oxidized lipids, or polyphenols may lead to the appearance of neoallergens.[5,9] In particular, under certain heating conditions and in the presence of reducing carbohydrates, protein glycation by the Maillard reaction (MR) occurs. It has been reported that the covalent modification of the peanut allergen Ara h 1 by sugar molecules during roasting increases its immunoglobulin E (IgE)-binding properties and makes it less digestible,[10] whereas the allergenicity of hazelnut is reduced by roasting.[11] MR between Pru av 1, the major allergen from cherry, and glucose or ribose induces a strong decrease in its IgE-binding capacity.[12] Similarly, conjugation of tropomyosin with ribose suppresses its specific IgE-binding ability and, although in this case the digestibility of the protein is impaired with the progress of MR, the lowered antigenicity remains after digestion.[13]

Proteins in egg white denature and become insoluble at relatively low temperatures, which poses a limitation to egg thermal processing. Spray-drying is preferred to avoid denaturation of native egg white proteins.[14] However, MR between glucose (about 4% of the solids in egg white) and the amino groups of proteins occurs during and after drying, so sugared spray-dried egg white can develop undesirable color and taste even after short periods of storage at ambient temperature. Because of this, in industrial practice, egg white is submitted to a desugaring process step, prior to the conventional spray-drying process, to protect the product against MR during heating and storage.[15]

During the drying process and subsequent storage, and depending on the efficiency of the desugaring process, it cannot be excluded that the free amino groups of egg proteins are glycated as a consequence of MR, which could change their conformation and thus their allergenicity. However, although the effect of heat treatment on the allergenic properties of egg proteins has been the focus of a few studies,[16−19] there are no previous reports on the influence of MR on their allergenic potential. Therefore, the present study focuses on the effect of heating and MR on the digestibility and IgG- and human IgE-binding properties of the egg white allergens OVA and OM.

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Table 1. Specific IgE Levels toward Egg White and Yolk, OVA, and OM of the Sera Used in the Study and Ages of the Patients[a]

serum	age	IgE to egg white (kU/L)	IgE to egg yolk (kU/L)	IgE to OVA (kU/L)	IgE to OM (kU/L)
1	3			36.3	23.5
2		26.8	2.69	32.1	29.7
3	12	>100	85.7	78.9	69.2
4	3	64.7	14	78.1	19.0
5	4	56.8		57.1	44.9

[a] Sera 1, 3, and 4 were used for OVA inhibition ELISA, whereas sera 2, 3, and 5 were used in OM inhibition ELISA.

MATERIALS AND METHODS

Hen egg OVA grade VI and ovoinhibitor-depleted OM were from Sigma-Aldrich (St. Louis, MO).

Heat Treatments and Glycation by MR. OVA or OM was dissolved in Milli-Q water to 5 mg/mL at pH 7.0. OVA and OM solutions were heated in a water bath at 65 °C for 30 min and at 90 °C for 15 min, simulating common heat treatments such as pasteurization and cooking. After the heat treatments, the pH was readjusted to 7.0 and the solutions were freeze-dried.

For MR, OVA or OM was dissolved in 0.1 M phosphate buffer at pH 7.2 and mixed with D-(+) glucose from Sigma-Aldrich, at a protein/glucose ratio of 1:0.05 (w/w). This proportion was chosen because egg white contains about 9.7−10.6% of proteins and 0.4−0.9% carbohydrates, with glucose accounting for 98% of the total free carbohydrates.[15] OVA and OM solutions were freeze-dried and stored for 48 and 96 h in desiccator at 50 °C and 0.65 water activity, by using a saturated solution of potassium iodide, to favor MR. After storage, the products were reconstituted in distilled water to a protein concentration of 1 mg/mL, and free glucose was removed using ultrafiltration devices of 3000 Da cutoff (Millipore, Bedford, MA) and centrifugation at 2900*g* for 45 min and 4 °C, until no color change was detected at 490 nm in the ultrafiltration permeates after the addition of the phenol−sulfuric acid reactive. It was checked that no loss of protein occurred after each ultrafiltration by measuring the absorbance at 280 nm using a Beckman Du 800 spectrophotometer (Fullerton, CA).[20]

The progress of MR was followed in the glycated samples by determining the absorbance at 420 nm, using a Beckman Du 800 spectrophotometer, and the free amino groups, using the 2,4,6-trinitrobenzenosulfonic acid method (TNBS, Sigma).[20] Finally, the samples were frozen and freeze-dried.

In Vitro Gastroduodenal Digestion. In vitro digestions were performed following refs 21 and 22. Aliquots were taken after 10, 20, and 60 min of gastric digestion of native, heated, and glycated OVA and OM and used for SDS-PAGE, RP-HPLC, and ELISA analyses. Gastric digests after 60 min of digestion were readjusted at pH 6.5 and subjected to duodenal digestions. In the case of OVA, Corolase PP (Rohm, Darmstadt, Germany) at 1:25 enzyme substrate ratio (w/w) was used instead of trypsin and α-chymotrypsin. Aliquots were also taken after 30 and 60 min of duodenal digestion for SDS-PAGE, RP-HPLC, and ELISA. In vitro gastric and duodenal digestions and subsequent analyses were carried out at least in duplicate.

SDS-PAGE. Analysis by SDS-PAGE was performed in a PhastSystem Electrophoresis equipment, using precast homogeneus gels 20% (GE Healthcare, New York) and PhastGel SDS buffer strips (Amersham Biosciences, Uppsala, Sweden), following the manufacturer's instructions for the electrophoretic and Coomasie staining conditions. Samples were dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 2.5% SDS and 10 mM EDTA, and heated at 95 °C for 10 min in the absence (nonreducing conditions) or presence (reducing conditions) of 5% 2-β-mercaptoethanol (β-ME).

RP-HPLC. RP-HPLC analysis was performed using a Waters 600 HPLC instrument (Waters, Milford, MA) and a 250 mm × 4.6 mm Widepore C18 column (Bio-Rad, Richmond, CA). Operating conditions were as follows: column at room temperature; flow rate, 1 mL/min; injection volume, 40 μL; solvent A, 0.37 mL/L TFA in Milli-Q water; and solvent B, 0.27 mL/L TFA in HPLC grade ACN. A linear gradient of solvent B in A, from 0 to 60% in 60 min, followed by 60% B for 30 min, was used. Absorbance was recorded at 220 nm with a Waters 2487 λ dual detector. The software was Empower 2000 system data (Waters).

IgG Binding by Direct ELISA and Human IgE Binding by Inhibition ELISA. The IgG binding of native, heated at 90 °C, and 96 h glycated OVA and OM and their gastroduodenal hydrolysates at different times was evaluated by direct ELISA. Polystyrene microtiter plates (Corning, Cambridge, MA) were used as a solid support, and single wells were coated with 50 μL of antigen at 2.4 μg/mL in 0.01 M phosphate-buffered saline solution (PBS), pH 7.4, and incubated overnight at 4 °C. Plates were washed with PBS containing 0.05% Tween 20 (PBST) using a microplate washer (Nunc, Roskilde, Denmark). This washing system was used after each incubation step. Residual free binding sites were blocked with PBS containing 2.5% Tween 20 for 2 h at room temperature. Then, the plates were incubated for 1 h at room temperature with 50 μL per well of an antibody specific for each protein: commercial polyclonal rabbit anti-OVA IgG (Gene Tex, Inc., Irvine, CA) or rabbit anti-OM IgG conjugated with horseradish peroxidase (HRP) (Immune Systems Ltd., ISL, Paignton, U.K.) diluted in PBST. A solution of freshly prepared *o*-phenylenediamine dihydrochloride (OPD, Palex Medical, Barcelona, Spain), in Milli-Q water containing 0.41% H_2O_2 (Panreac, Barcelona, Spain), was added following the conditions of the manufacturer. Plates were incubated for 30 min at room temperature, and the reaction was stopped by adding 50 μL per well of 0.5 M H_2SO_4. Optical densities were read at 492 nm on an automated ELISA plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). ELISA determinations were carried out in triplicate and measurements averaged. A blank without antigen, the negative control with antigen (OVA or OM) but without antibody, and positive controls, commercial OVA or OM at different concentrations, were included in each plate. IgG-binding data were statistically processed using one-way ANOVA. In all cases, p values ≤ 0.05 were considered to be statistically significant.

Human IgE binding of native, heated at 90 °C, or 96 h glycated OVA and OM and their hydrolysates at different times was evaluated by inhibition ELISA following ref 23 with slight variations: OPD was used as substrate, and optical densities were read at 492 nm. The sera used are described in Table 1.

Calculations for IgE binding were as follows. The absorbance recorded from each sample was converted into an inhibition percentage using this equation adapted from Schmitt:[24]

$$\%\ \text{inhibition} = \left(1 - \frac{A2 - A1}{A0 - A1}\right) \times 100$$

A2 is the absorbance for a given inhibitor concentration, A1 the minimum signal (maximum inhibition), and A0 the maximum signal (minimum inhibition). The GraphPad Prism package (GraphPad Software Inc., San Diego, CA) was used to adjust the inhibition percentages, depending on the concentration (X), to dose−response sigmoid equations with variable slope according to

$$EC_{50} = A + \frac{B - A}{(1 + 10^{(\log EC_{50} - X) \times k})}$$

where A and B are, respectively, the bottom (lowest value) and the top (highest value) of the sigmoidal curve; X is the decimal logarithm of the

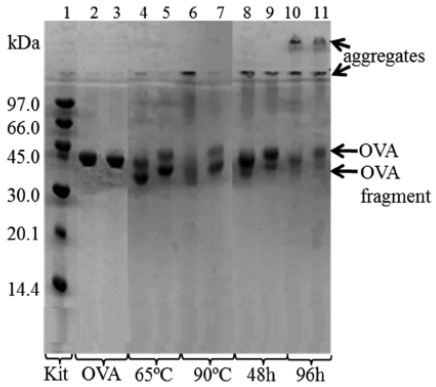

Figure 1. SDS-PAGE patterns, under nonreducing (lanes 2, 4, 6, 8, and 10) and reducing conditions (lanes 3, 5, 7, 9, and 11), of native, heated, and glycated OVA. Lanes: 1, standard proteins; 2 and 3, native OVA; 4 and 5, OVA heated at 65 °C for 30 min; 6 and 7, OVA heated at 90 °C for 15 min; 8 and 9, OVA glycated for 48 h; 10 and 11, OVA glycated for 96 h.

protein concentration; k is the slope; and EC_{50} is the effective sample concentration for 50% of the maximum binding. The EC_{50} for each sample was calculated and normalized as a percentage of the value obtained for the commercial OVA or OM, included in each plate as a control, to normalize the results between plates:

$$\%\ \text{of IgE binding} = \frac{EC_{50}(\text{control})}{EC_{50}(\text{sample})} \times 100$$

The IgE-binding percentages obtained for each sample were statistically processed using a two-way ANOVA (processing and in vitro digestion) and repeated measurements (three patients' sera), followed by Bonferroni post-tests. p values of ≤ 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Effects of Heating and MR on the Digestibility and IgG and IgE Binding of OVA. Figure 1 shows the SDS-PAGE pattern of native, heated, and glycated OVA under nonreducing and reducing conditions. In general terms, OVA migrated at a lower molecular mass under nonreducing conditions compared to reducing conditions. Native OVA appeared in the SDS gels as a band of ~45 kDa (Figure 1, lanes 2 and 3). After heating at 65 °C for 30 min or at 90 °C for 15 min, in addition to native OVA, a second band with lower molecular mass was visible (Figure 1, lanes 4−7). A band with the same mobility was also detected following digestion with pepsin (see below). High molecular mass aggregates (>66 kDa) were formed upon heating (Figure 1, lanes 4 and 6), particularly at 90 °C, which exceeds the denaturation temperature of the protein of 78.3 °C.[25] These aggregates seemed to be stabilized mainly by disulfide bonds, because they were reduced in the presence of β-ME (Figure 1, lanes 5 and 7).

Incubation of OVA with glucose led to an increase in color and a decrease in free amino groups, which indicated the development of MR. These changes were particularly evident during the first 48 h, with smaller differences afterward (Table 2). Kato et al.[26] reported that 50% of the amino groups of OVA are blocked after 2−4 days of storage with glucose (1:1, w/w) at 50 °C and 0.65 a_w.

The SDS-PAGE patterns obtained under nonreducing conditions of the glycated OVA samples provided evidence for the aggregation of OVA, with bands of molecular mass higher than 66 kDa, as well as bands retained in the stacking gel and in the boundary between the stacking and the resolving gel (Figure 1, lanes 8 and 10). Covalent aggregation of OVA was more noticeable after 96 h of incubation with glucose, despite the fact that changes in color and accessibility of free amino groups were not very important after 48 h. In addition, these aggregates were only partially reduced with β-ME (Figure 1, lanes 9 and 11), which pointed out that, under these conditions, MR of OVA with glucose led to cross-linking of OVA molecules by both disulfide and nonreducible covalent bonds, which is in agreement with previous results.[27] In fact, it has been reported that glucose strongly promotes OVA polymerization, which is attributed to a rapid degradation of the Amadori compounds into highly reactive MR products.[26]

Table 2. Color Development at 420 nm and Free Amino Groups of OVA and OM, Native and Glycated with Glucose at 50 °C and 0.65 a_w, during 48 or 96 h

sample	420 nm	% free NH_2
native OVA	0.0192	100
OVA 48 h	0.0237	60
OVA 96 h	0.0274	50
native OM	0.0117	100
OM 48 h	0.0251	41
OM 96 h	0.0403	5

It should be noted that the structural changes induced by heat treatment and MR led to the appearance of wider and lower retention time peaks for the heated and glycated OVA by RP-HPLC, as shown in the chromatograms of the undigested samples in Figure 2a.

In vitro gastric digestion of native OVA led to a SDS-PAGE pattern with two hydrolysis fragments, ~40 and ~4 kDa (Figure 3, lanes 2−4). The highest molecular mass degradation product, the mobility of which coincided with that of the band detected after heat treatment at 65 and 90 °C, probably corresponded to the C-terminal fragment Ala23−Pro385.[22,28] Intact OVA and its fragments were clearly visible in the gels after 60 min of digestion, confirming that OVA is very stable to pepsin at E/S close to 1:20 w/w.[22,29] The samples obtained after 60 min of in vitro gastric digestion with pepsin were further subjected to a process mimicking duodenal digestion with Corolase PP. In agreement with previous results,[22,28] the band of native OVA rapidly decreased, whereas the fragment of ~40 kDa persisted for at least 60 min (Figure 3, lanes 8 and 9).

No differences in susceptibility to digestion were found between native OVA and OVA heated at 65 °C for 30 min (data not shown). However, OVA heated at 90 °C for 15 min was much more prone to degradation: HPLC profiles showed an increased amount of peptides (Figure 2), whereas no intact OVA or its ~40 kDa fragment were found in SDS-PAGE after 10 min of in vitro gastric digestion (Figure 3, lanes 5−7). The lower molecular mass products of ~4 kDa detected after gastric digestion completely disappeared during duodenal in vitro digestion (Figure 3, lanes 10 and 11). This agrees with the results of Takagi et al.,[28] who found that heating at 100 °C for 5 min significantly accelerates proteolysis of OVA.

OVA glycated for 48 h was digested similarly to native OVA (data not shown). However, in the case of OVA glycated for

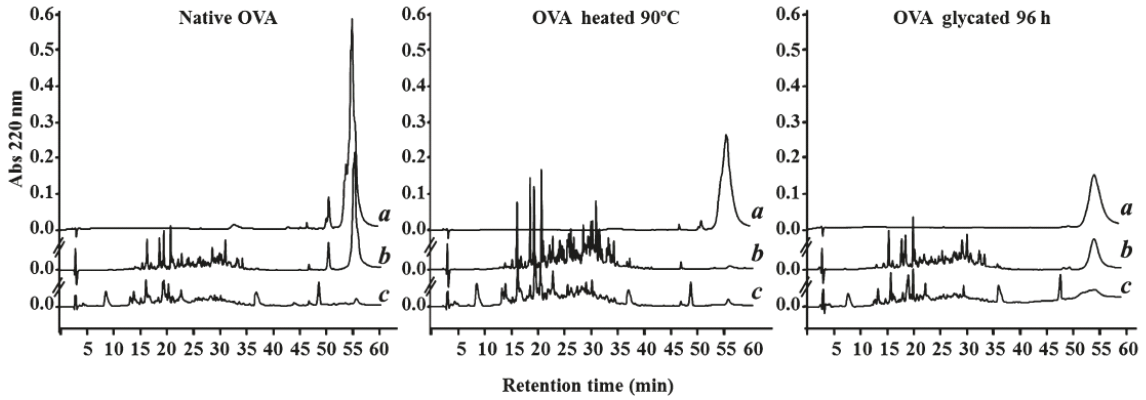

Figure 2. RP-HPLC analyses of (a) native, heated (90 °C, 15 min), and glycated (96 h) OVA and their respective (b) in vitro gastric digests after 60 min and (c) in vitro duodenal digests after 30 min.

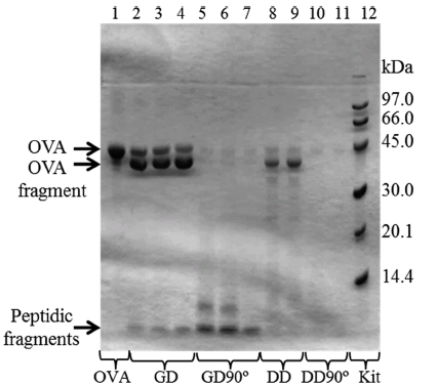

Figure 3. SDS-PAGE patterns under reducing conditions of native and heated OVA after gastroduodenal digestion. Lanes: 1, native OVA; 2−4, in vitro gastric digests (GD) of native OVA after 10, 20, and 60 min; 5−7, GD of heated OVA (90 °C for 15 min) after 10, 20, and 60 min; 8 and 9, in vitro duodenal digests (DD) of native OVA after 30 and 60 min; 10 and 11, DD of heated OVA (90 °C for 15 min) after 30 and 60 min; 12, standard proteins.

96 h, the high molecular weight aggregates formed during MR resisted gastric digestion, and they were still visible after duodenal digestion (results not shown). Furthermore, 96 h glycated OVA was still detected as a wide peak after duodenal digestion (Figure 2). Glycation has been reported to decrease the digestibility of allergens, such as those from wheat, squid, peanuts, or milk, due to the formation of Maillard-type protein aggregates.[10,13,30−32]

Panels a and b of Figure 4 show, respectively, the IgG and IgE binding of native, heated, and glycated OVA as well as those of their in vitro gastric and duodenal digests. The human sera used are described in Table 1. Whereas it should be noted that allergenic epitopes only partially overlap antigenic determinants, the reactivities against IgG and IgE after processing and in vitro digestion followed a similar trend. The IgG binding of native OVA was significantly reduced after 10 min of gastric digestion, and it decreased slowly with further degradation. The resistance of native OVA to hydrolysis by pepsin and to subsequent hydrolysis by pancreatic enzymes led to gastric and duodenal hydrolysates that retained noticeable IgG- and IgE-binding properties (around 20% of the IgG-binding activity and 30% of the IgE-binding activity were kept at the end of duodenal digestion, Figure 4).

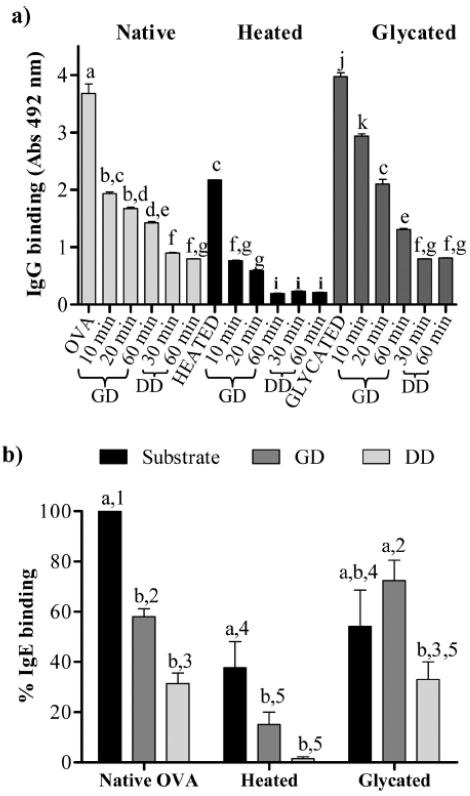

Figure 4. (a) Direct ELISA response (Abs 492 nm) against rabbit anti-OVA IgG of native, heated (90 °C for 15 min), and glycated OVA (96 h) and their in vitro gastric (GD) and duodenal digests (DD) after different digestion times. Error bars correspond to the mean ± SD. Different letters above the bars represent significant differences ($p < 0.05$) ($n = 3$). (b) Inhibition ELISA response against IgE from individual sera of allergic patients of native, heated (90 °C, 15 min), and glycated (96 h) OVA and their GD after 60 min and DD after 30 min. Results are presented as the mean ± SD of three sera ($n = 3$). Different letters indicate significant differences ($p < 0.05$) within each treatment regarding the digestion time, whereas different numbers indicate significant differences ($p < 0.05$) regarding the treatment.

Significant reductions in IgG and IgE binding (to approximately 40 and 60% of the original values, respectively, Figure 4, panels a and b, respectively) were found when OVA was heated at 90 °C for 15 min, which is in agreement with Honma et al.,[33] who reported important reductions in IgG and IgE binding in

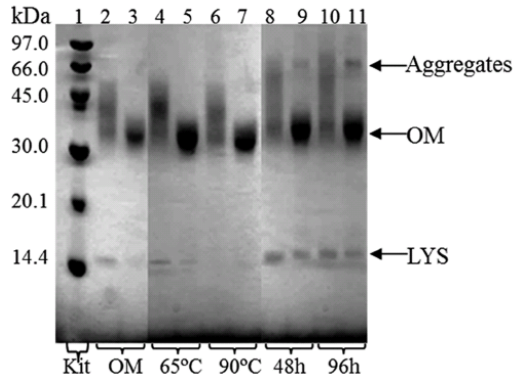

Figure 5. SDS-PAGE patterns, under nonreducing (lanes 2, 4, 6, 8, and 10) and reducing conditions (lanes 3, 5, 7, 9, and 11), of native, heated, and glycated OM. Lanes: 1, standard proteins; 2 and 3, native OM; 4 and 5, OM heated at 65 °C for 30 min; 6 and 7, OM heated at 90 °C for 15 min; 8 and 9, OM glycated for 48 h; 10 and 11, OM glycated for 96 h.

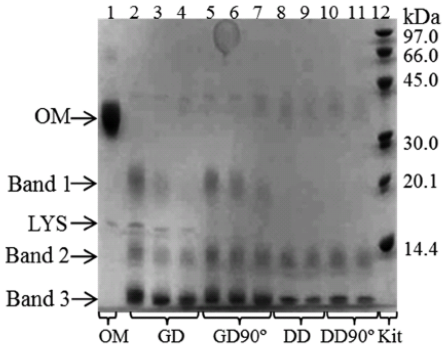

Figure 6. SDS-PAGE patterns under reducing conditions of native and heated OM after gastroduodenal digestion. Lanes: 1, native OM; 2−4, in vitro gastric digests (GD) of native OM after 10, 20, and 60 min; 5−7, GD of heated OM (90 °C for 15 min) after 10, 20, and 60 min; 8 and 9, in vitro duodenal digests (DD) of native OM after 30 and 60 min; 10 and 11, DD of heated OM (90 °C for 15 min) after 30 and 60 min; 12, standard proteins.

heat-denatured OVA (100 °C for 3 min). Similarly, Mine and Zhang[19] described that a heat treatment at 95 °C for 15 min lowers the binding of OVA to human IgE. Kim et al.[17] also found that human IgE does not recognize well OVA heated at ≥80 °C, whereas mouse IgG retains more binding capacity under those conditions. On the other hand, and in accordance with its higher susceptibility to proteolysis, the in vitro gastric and duodenal digests of OVA heated at 90 °C for 15 min exhibited the lowest IgG- and IgE-binding capacities. This is consistent with the observation that most patients with egg allergy tolerate heated egg.[16,34]

With the human sera tested, OVA glycated for 96 h showed a significantly lower IgE-binding capacity than native OVA (Figure 4b), in contrast with the IgG-binding results (Figure 4a). A strong decrease in the IgE-binding capacity of several allergenic proteins has been found as a consequence of MR with certain monosaccharides, which is attributed to irreversible changes in protein structure that result in the loss of conformational epitopes.[12,13] However, because of its lower susceptibility to in vitro digestion, glycated OVA retained more IgG- ($p \leq 0.05$) and similar IgE-binding activity than native OVA after pepsin hydrolysis. The formation, as a result of MR, of high molecular weight aggregates could have exerted a protecting effect, reducing the accessibility to the enzyme of certain epitopes. IgG and IgE binding significantly decreased after duodenal digestion of the glycated protein ($p \leq 0.05$), although around 20% of the IgG- and 30% of the IgE-binding responses, which are levels similar to those of the Corolase-digested native protein, were kept after 30 min (Figure 4). In agreement with the present results, it has been reported that the decreased in vitro protein digestibility that results from MR of wheat proteins during baking gives rise to persistent IgE-binding components, which led to a higher potential allergenicity than that of the untreated dough.[30] In the case of other allergens, such as tropomyosin, glycation considerably decreases its reactivity toward human IgE, so that, even though the digestibility of the protein by pepsin also decreases, a lower IgE binding remains after digestion.[13]

Effects of Heating and MR on the Digestibility and IgG and IgE Binding of OM. OM has a molecular mass of 28 kDa; however, in SDS-PAGE under nonreducing conditions it appeared as a diffuse group of bands from 30 to 40 kDa (Figure 5, lane 2). This overweight estimation of OM is attributed to a low SDS to protein binding ratio, because OM is highly glycosylated and SDS does not bind to the carbohydrate chains.[35] OM is composed of 186 amino acids ordered in three domains internally stabilized by disulfide bonds[36] and, therefore, when OM was treated under reducing conditions, a narrower OM band could be seen (Figure 5, lane 3). No differences were found between the patterns of native and heated OM because of its high stability toward heat treatment.[16] The SDS-PAGE analysis of OM also showed a band of hen egg lysozyme (LYS), present due to an incomplete purification of the commercial product.[37]

OM continuously developed brown color during the 96 h of glycation and, in addition, there was a considerable reduction in the number of available amino groups (Table 2). This almost complete loss of reactivity of the free amino groups, as measured by the TNBS method, was not likely to correspond exclusively to the formation of linkages with the reducing end of the glucose but also to protein aggregation. Evidence for aggregate formation was obtained from the SDS-PAGE analysis, which showed, under nonreducing conditions, the presence of higher molecular mass products, between 30 and 66 kDa, which were more abundant in the sample subjected to MR for 96 h than for 48 h (Figure 5, lanes 8 and 10). When the glycated samples were treated under reducing conditions (Figure 5, lanes 9 and 11), the persistence of a band of ~50 kDa indicated that the OM aggregates were not completely stabilized by disulfide bonds but also by other covalent protein cross-links. The patterns of the glycated samples under reducing conditions also showed that the main OM band exhibited a slightly higher molecular mass, possibly as a result of the attachment of glucose molecules.

During the in vitro gastric digestion, the SDS-PAGE pattern showed the disappearance of OM within the first 10 min with the formation of three new bands with molecular masses of ~18, ~13, and <3 kDa (named, respectively, bands 1, 2, and 3 in Figure 6). A similar behavior was described by Matsuda et al.[38] and Kovacs-Nolan et al.,[39] who studied the in vitro gastric digestion of OM under different conditions. The 18 kDa band was hydrolyzed, whereas the other two bands remained after duodenal digestion. It is known that the trypsin inhibitory activity of OM is reduced by pepsin digestion,[40] but residual activity might help to maintain OM peptide fragment integrity.[39] The aggregates formed by MR quickly disappeared within 10 min of gastric digestion (data not shown). As illustrated by the SDS-PAGE and RP-HPLC analyses, heated and glycated OM had an

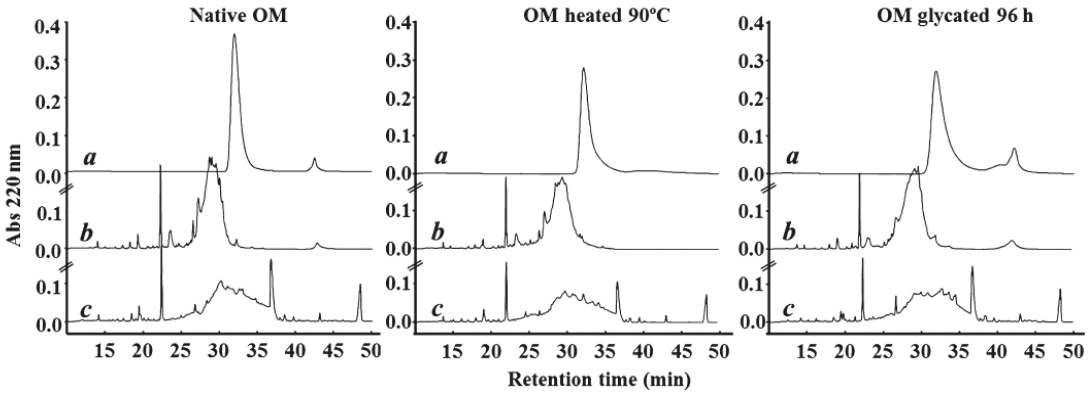

Figure 7. RP-HPLC analyses of (a) native, heated (90 °C, 15 min), and glycated (96 h) OM and their respective (b) in vitro gastric digests after 60 min and (c) in vitro duodenal digests after 30 min.

in vitro digestion behavior similar to that of native OM (Figures 6 and 7).

Native OM showed a high reactivity toward IgG that significantly decreased upon digestion, although OM retained, approximately, 60% of its IgG-binding activity at the end of the duodenal phase (Figure 8a). IgG binding of OM heated to 90 °C and that of OM glycated for 96 h were similar to that on native OM and, in agreement with the similarities found in their behavior toward in vitro digestion, they all gave rise to peptide fragments that contained epitopes recognizable by IgG.

After in vitro gastric and duodenal digestions, the immunoreactivity of native OM toward IgE from the egg-allergic patients studied was reduced to a greater extent than that toward IgG (Figure 8b), which illustrates that the recognition by human and animal antibodies varies due to differences in the species and the sensitization routes.[19,42] It has been reported that the IgE-binding activity to pepsin-digested OM might be useful to identify subjects that are unlikely to outgrow egg white allergy.[43] Nevertheless, epitopes recognizable by human IgE were kept even after duodenal digestion, in accordance with previous findings showing that IgE-binding activity of OM is reduced, but not eliminated, upon digestion.[38,39] Takagi et al.,[41] who studied the allergenic potential of pepsin-digested OM, found that the fragments resistant to gastric digestion contained IgE-binding epitopes and that 21% of the allergic patients examined retained IgE-binding capacity to the small 7 and 4.5 kDa fragments.

Heating at 90 °C for 15 min significantly reduced the IgE-binding activity of OM to, approximately, 50%. This is in agreement with Mine and Zhang,[19] who described that a heat treatment at 95 °C for 15 min significantly lowers the binding of OM to both rabbit IgG and human IgE, although, under those conditions, heating has much less effect on OM structure than on OVA structure. Overall, the results from these authors pointed out that anti-OVA IgE recognizes mainly sequential epitopes and anti-OM IgE recognizes both conformational and sequential epitopes.[19] In any case, and as was described for the proteolysis fragments,[40] the reactivity of IgE from egg-allergic patients toward native or heated (100 °C, 30 min) OM varies depending on their individual susceptibility.[44]

According to our results, glycation of OM for 96 h significantly increased binding to IgE from the sera studied (Figure 8b), either because new epitopes were created or because glucose favored IgE recognition. Different results have been reported regarding

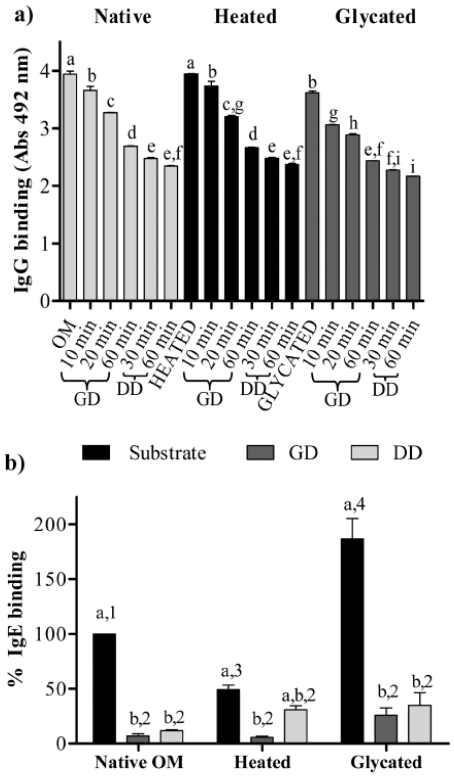

Figure 8. (a) Direct ELISA response (Abs 492 nm) against rabbit anti-OM IgG of native, heated (90 °C for 15 min), and glycated OM (96 h) and their in vitro gastric (GD) and duodenal digests (DD) after different digestion times. Error bars correspond to the mean ± SD (n = 3). Different letters above the bars represent significant differences (p < 0.05). (b) Inhibition ELISA response against IgE from individual sera of allergic patients of native, heated (90 °C, 15 min), and glycated (96 h) OM and their GD after 60 min and DD after 30 min. Results are presented as the mean ± SD of three sera (n = 3). Different letters indicate significant differences (p < 0.05) within each treatment regarding the digestion time, whereas different numbers indicate significant differences (p < 0.05) regarding the treatment.

the influence of MR on the IgE-binding activity of food allergens. Thus, Maleki et al.[10] described an increase in the IgE-binding activity of peanut protein after glycation with different sugars, including glucose, and they attributed this to the formation of novel IgE-binding sites due to covalent modifications of the protein during MR. The opposite effect has been described on

Pru av 1 glycated with glucose or ribose by MR,[12] and it was hypothesized that reactive carbonyl intermediates could have induced a loss of conformational epitopes by modifying the nucleophilic amino acid side chains of the protein.

Despite the differences encountered in the IgE-binding activity of the native, heated, or glycated OM, their digestion products exhibited similar IgE responses (Figure 8b). This is probably a result of the similarities found in the susceptibility to proteolysis and in the hydrolysis pattern in the three cases that could have led to a comparable destruction of the allergenic epitopes.

This paper shows that heat treatment and glycation by MR have an influence on the potential allergenicity of some of the main egg white proteins but that the effect of processing on antibody binding and susceptibility to proteolysis of the allergens may depend on the intensity of the treatments and their intrinsic resistance to denaturation and digestive enzymes. The human IgE-binding studies, conducted with sera from three patients, showed that heat treatment (90 °C, 15 min) significantly reduced the IgE binding of both OVA and OM, whereas MR (with 1:0.05 glucose, w/w, for 96 h, at 50 °C and 0.65 water activity) reduced the IgE binding to OVA, but increased the binding to OM, a protein more resistant to denaturation. On the other hand, glycation impaired OVA digestibility, particularly by gastric enzymes, but did not affect the digestibility of OM, the native form of which, unlike that of OVA, is normally quickly degraded by pepsin.

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ABBREVIATIONS USED

OVA, ovalbumin; OM, ovomucoid; MR, Maillard reaction; GD, gastric digests; DD, duodenal digests.

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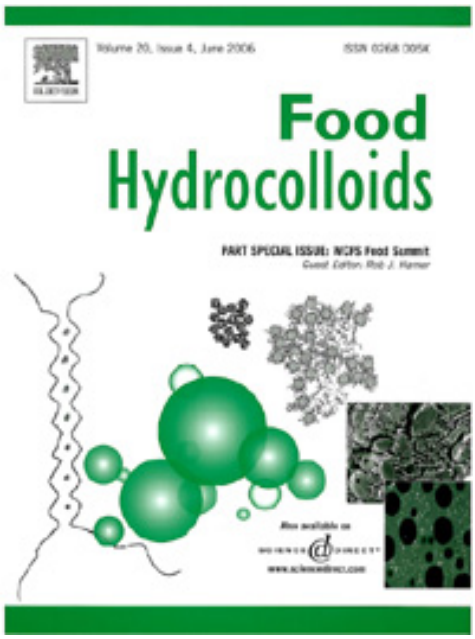

Food Hydrocol. (Enviado)

Intestinal stability of egg allergens in the presence of polysaccharides

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Food Hydrocolloids

Intestinal Stability of Egg Allergens in the Presence of Polysaccharides

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Keywords: matrix effect, digestion, egg allergens, polysaccharides, food allergy, IgE-binding.

Abstract

Egg proteins are ubiquitous in many foods because of their high nutritional value and functional properties, but some of them are also important allergens, the ovalbumin being the most abundant and the ovomucoid considered the immunodominant. On the other hand, polysaccharides such as pectin, gum arabic and xylan are functional biopolymers commonly used in the food industry. It is known that the interactions between proteins and polysaccharides within the food matrix may affect their stability in the intestine and therefore modify its ability of triggering an immune response. Thus, the aim of the present work was to study the *in vitro* gastrointestinal stability of ovalbumin and ovomucoid in the presence of pectin, gum arabic and xylan as well as the IgE-binding of the digests. Solutions of ovalbumin or ovomucoid and pectin, gum arabic or xylan were *in vitro* digested by using a model that mimics physiological conditions. Gastric and duodenal digests were analyzed by SEC and RP-HPLC. Also, the specific human-IgE binding capacity of the duodenal digests was assessed by inhibition ELISA by using sera from egg-sensitized patients. Digestion of both proteins in the presence of polysaccharides was diminished when compared with that of native proteins. Regarding IgE-binding, the duodenal digests obtained in the presence of polysaccharides retained more IgE-binding than the native protein digests. From the results obtained, it was concluded that the interaction of ovalbumin and ovomucoid with pectin, gum arabic and xylan hampered gastro duodenal digestion with a consequent increase on the *in vitro* allergenicity.

1. Introduction

Egg proteins are broadly used in the food industry due to their unique functional properties such as emulsifying, foaming and gelling. In addition, the high biological quality of egg proteins adds more value to the food products that include egg or its constituents (yolk, albumen) as ingredients (Kovacs-Nolan, Phillips, & Mine, 2005; Mine, 2002). However, egg is an important source of allergens, which are mainly focused in the egg white, with ovalbumin (OVA or Gal d 2) and ovomucoid (OM or Gal d 1) being the major ones (Mine & Yang, 2008). Among food allergies, allergy to egg is, together with peanut or milk, the most common in children and infants with a prevalence that varies, between 7.9 and 10% (Osborne, Koplin, Martin, Gurrin, Lowe, Matheson, et al., 2011) although it differs depending on the study (Sicherer, 2011)

Among the conditions required for food proteins to trigger an allergic reaction is their ability to keep the integrity of their allergenic determinants through the gastrointestinal tract. To date, most of the studies dealing with the influence of the gastro duodenal digestion on the potential allergenicity of foods have been carried out on isolated proteins (Moreno, 2007). However, exposure of allergic individuals to pure allergens is rare and, in fact, the stability of proteins to digestion can be altered in the presence of various components that form part of the food matrix (Teuber, 2002).

Thus, several studies have demonstrated that the presence of soluble polysaccharides, commonly used in the preparation of a wide range of foods, namely ice-creams, jams, jellies or fruits juices, as stabilizers, thickeners and emulsifiers, can reduce protein digestibility of food proteins, being the increase of mixture viscosity, the interactions between the two types of macromolecules, and the inhibition of enzymatic activity often mentioned to explain this observation (Mouecoucou, Fremont, Sanchez, Villaume, & Mejean, 2004a; Mouecoucou, Sanchez, Villaume, Marrion, Fremont,

Laurent, et al., 2003). Polovic, Pjanovic, Burazer, Velickovic, Jankov and Velickovic (2009) reported the existence of protective matrix effect on the digestion of pectin-rich crude extracts of various fruits. Pectin forms gels in the conditions present during *in vivo* and *in vitro* pepsin digestion and provides a physical obstacle to the mobility of pepsin and protein substrate in the reaction mixture. Protective effects of structural polysaccharides of plant origin on the *in vitro* digestion stability of peanut allergens and β-lactoglobulin by pepsin and trypsin have been reported (Mouecoucou et al., 2004a; Mouecoucou, Villaume, Sanchez, & Mejean, 2004b). Furthermore, polysaccharides can interact with the digestion products to form complexes that reduce the antigenicity of milk and peanut proteins (Mouecoucou, Fremont, Villaume, Sanchez, & Mejean, 2007). Interestingly, although the digestibility of these allergens is impaired, some of them show less IgG/IgE binding capacity that was attributed to the masking of epitopes because of the interaction with the polysaccharides (Mouecoucou at al., 2004a; Mouecoucou et al., 2007). Unfortunately, other studies did not go further than the digestibility assessment to include assays for potential allergenicity (Mouecoucou et al., 2003; Polovic et al., 2009)

Regarding egg allergens, and although they have been intensively investigated, there are only a few studies on the effect of carbohydrate-protein interactions on their digestibility and immunogenicity (Jiménez-Saiz, Belloque, Molina, & López-Fandiño, 2011a). Typically, foods contain proteins and polysaccharides as complex multicomponents mixtures, in many cases interacting as mixed biopolymers (Dickinson, 1998). Therefore, the aim of the present work was to investigate the *in vitro* gastrointestinal behaviour and the resulting human IgE-binding of the main egg allergens, OVA and OM, in the presence of the polysaccharides: pectin, gum arabic and xylan.

2. Materials and methods

2.1 Protein-polysaccharide mixes

Hen egg OVA grade VI, ovoinhibitor-depleted OM, high methylated pectin (P), gum arabic from acacia tree (G) and xylan from beechwood (X) were from Sigma-Aldrich (St. Louis, MO). Proteins and polysaccharides were dissolved separately in 0.1 M NaCl (20 mg/mL) and pH was adjusted to 3. The polysaccharides were heated at 60ºC for 15 min to improve their solubility. Then, OVA and OM solutions were mixed with each polysaccharide (1:1, w/w) and shaked gently overnight at room temperature (RT).

2.2 Gastric and duodenal digestions

The digestibility of OVA and OM and their mixtures with each polysaccharide (P, G, X) was studied using an *in vitro* model system in two steps, which imitates gastric and duodenal digestion *in vivo* (Martos, Contreras, Molina, & López-Fandiño, 2010). Gastric digestion was performed in simulated gastric fluid (SGF, 35 mM NaCl) at pH 2.0, for 60 min at 37ºC, with 172 U/mg of porcine pepsin (EC 3.4.23.1**,** 3210 U/mg protein, Sigma-Aldrich). The reaction was stopped by rising pH to 7 with 1M $NaHCO_3$.

Duodenal digestions were performed by using the 60 min gastric digests adjusted to pH 7, as described above, with the addition of: 1 M $CaCl_2$, 0.25 M Bis-Tris pH 6.5 and 0.125 M bile salts. Porcine pancreatic lipase (EC 232-619-9), colipase (EC 259-490-1), trypsin (EC 232-650-8. 10100 BAEE units/mg protein) and α-chymotrypsin (EC 232-671-2: 55 units/mg protein) from Sigma-Aldrich were used. Aliquots were taken after 60 min of gastric digestion and 30 min of duodenal digestion. Duplicate digestions were conducted for each condition.

2.3 Reverse phase high-performance liquid chromatography (RP-HPLC)

The RP-HPLC analysis was performed using a Waters 600 HPLC instrument (Waters, Milford, MA) and a 250 mm X 4.6 mm Widepore C18 column (Bio-Rad, Richmond, CA). Operating conditions were as follows: column at RT; flow rate at 1 mL/min; injection volume at, 50 μL; solvent A (0.37 mL/L TFA in Milli-Q water); and solvent B (0.27 mL/L TFA in HPLC grade acetonitrile). A linear gradient of solvent B in A, from 0 to 60% in 60 min, followed by 60% B for 30 min, was used. Absorbance was recorded at 220 nm with a Waters 2487 λ dual detector. The software Empower 2000 system data (Waters) was used.

2.4 Size exclusion chromatography (SEC)

The SEC analysis was performed using a Waters 600 HPLC instrument (Waters) and a 7.8 mm X 300 mm TSK-gel G2000SWxl column (Tosoh Bioscience, Tokyo, Japan). Operation conditions were as follows: column at RT, flow rate at 1 mL/min; injection volume at 30 μL. Samples were eluted in isocratic mode by using 0.1 M NaCl at pH 7. Absorbance was recorded at 220 nm with a Waters 2487 λ dual detector. The software Empower 2000 system data (Waters) was used. Fractions of interest were manually collected and frozen at -30ºC for further use.

2.5 Human IgE-binding

A total of 9 sera form children with clinical and allergic symptoms to egg were mixed in three different pools as shown in table 1. The sera were collected from the Maternal and Child Gregorio Marañón Hospital (Madrid, Spain). The patients´ specific seric IgE levels were determined by CAP (GE HealthCare).

Human IgE-binding of the duodenal digests was assessed by inhibition ELISA as previously reported (Jiménez-Saiz, Martos, Carrillo, López-Fandiño, & Molina, 2011b) with slight variations: commercial OVA and OM were used as a coating antigens diluted in 0.01 M phosphate buffer, pH 7.4 (PBS) to 80 μg/mL and polyclonal

rabbit anti-human IgE (A0094, Dako, Glostrup, Denmark) and polyclonal swine anti-rabbit immunoglobulins labelled with horseradish peroxidase (P0399, Dako) were used diluted 1:1000 and 1:2000 (v/v) respectively in PBS containing 0.05% Tween 20 (PBST). IgE-binding results were statistically processed following Jimenez-Saiz et al. (2011b). The EC50 of each sample was expressed as a mean ± standard error for n=2. Significant differences ($P<0.05$) were evaluated by one-way analysis of variance followed by post hoc multiple-comparison using Tukey´s test.

The ability to bind human-IgE of the fractions collected by SEC was assessed by indirect ELISA. Plates were coated with the samples non-diluted and incubated overnight at 4ºC. After overnight incubation, the plates were washed with PBST and incubated with sera from allergic patients. Following 2 hours of incubation, plates were washed and incubated with rabbit anti-human IgE. After a washing step, polyclonal swine anti-rabbit immunoglobulins labeled with horseradish peroxidase was added and incubated for 1 hour. Then, the plate was washed and the tyramide-biotin and streptavidin-HRP amplification system was used following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Waltham, MA). TMB was used as substrate and the reaction was stopped with sulfuric acid. The absorbance was measured at 450 nm in plate reader (Multiskan FC, Thermo Scientific, Finland).

3. Results and discussion

3.1 Digestibility of the mixtures of OVA and OM with polysaccharides

Gastric and duodenal *in vitro* digests of OVA mixed with P, G and X were analyzed by RP-HPLC. Gastric digestion of OVA, which elutes after 55 min, was hampered in the presence of the polysaccharides. Furthermore, the peak eluting at 51 minutes, which corresponds to the 40.1 kDa fragment, Ala_{23}-Pro_{385}, released by pepsin

action (Martos et al., 2010) and, in general terms, the peptide fragments released during the simulated gastric digestion showed higher intensity in the presence of the polysaccharides (results not shown), what suggests that the polysaccharides protected OVA and their digestion products from further hydrolysis by pepsin. In Figure 1, which shows the RP-HPLC profiles of the duodenal digests, the higher resistance to digestion of OVA in the presence of P, G and X is still observed, although the protector effect on the duodenal peptides was reduced. In the case of OM digested under simulated gastric conditions, there were no differences among the RP-HPLC profiles (data not shown). However, the digestibility of OM mixed with the polysaccharides under *in vitro* duodenal conditions was slightly reduced, especially in the presence of P and G, as it can be seen in the RP-HPLC analyses shown in Figure 2.

The establishment of non-covalent interaction between proteins and polysaccharides in solution depends on the conditions of pH, ionic strength and on the distribution of charged, hydrophobic, hydrogen bonding groups etc. (Dickinson, 1998). G and P, contain uronic acid, which is responsible for their anionic character, while X, composed of xylose, is neutral. At acidic pH, typical of the stomach (between 1.5-3) (Mackie & Macierzanka, 2010) proteins, below their isoelectric point, as it is the case of OVA and OM, can establish electrostatic associations with anionic polysaccharides. Such interactions, reinforced with non-ionic attractions, like hydrogen bonds, lead to complex formation between proteins and polysaccharides, while non-ionic interactions themselves are enough to establish associations between positively charged proteins and neutral oligomers, such as X, which may exert a protective effect towards proteolysis (Mouecoucou et al., 2004a). In addition, the ease of unfolding of the native structure of the protein or the changes induced because of the advance of digestion under acidic conditions could increase the chance of interaction (Patino & Pilosof, 2011). At neutral

pH, proteins and anionic polysaccharides usually carry a negative net charge, but electrostatic interaction may still take place between the negative charges on polysaccharides and positively charged regions on proteins, that could also be reinforced with non-ionic attractions (Dickinson, 1998; Mouecoucou et al., 2004a; Mouecoucou et al., 2004b). In our study, the protector effect during duodenal digestion was reduced, which could be attributed to the establishment of weaker interactions at the higher pH, as explained above, or to the action of bile salts that could compete with polysaccharides, because of their anionic character (Hofmann & Small, 1967).

Some authors have suggested that it is the interaction of polysaccharides with the digestion products —rather than the interaction with the undigested protein— what lowers digestion in the presence of P, G and X (Mouecoucou et al., 2004a; Mouecoucou, et al., 2003; Mouecoucou et al., 2004b). SEC analyses of gastric and duodenal digests of OVA and OM mixed with the polysaccharides are shown in Figures 3 and 4. With the advance of digestion, new peaks corresponding to hydrolysis products with lower size appeared in the chromatograms. In addition, peaks corresponding to higher molecular weight forms were detected in the presence of the polysaccharides (marked with * in Figures 3 and 4) that were not found in the OVA or OM samples used as controls (data not shown), nor in the mixtures of OVA and OM with the neutral polysaccharide X (Figures 3 and 4 c). Such peaks increased in intensity during gastric digestion, and new peaks (marked with ** in Figures 3 and 4) appeared, suggesting the binding of polysaccharides to peptide fragments released by pepsin. Upon simulated duodenal digestion, the first peak (marked with ** in Figure 3) was not longer detectable in any of the OVA-polysaccharides mixtures, while the area of the second peak decreased in OVA and OM mixed with P and G or remained unchanged in the mixtures with X. This suggests that P and G could have interacted electrostatically with

big or intermediate OVA fragments released by pepsin, as well as with smaller fragments resulting from pepsin action on OVA and OM that, in the latter case, resisted, at least partially, duodenal digestion. It is also likely that, under duodenal conditions, new associations were established between anionic polysaccharides and protein fragments containing basic amino acids released by trypsin (arginine and lysine). On the other hand, X probably established mainly non-ionic interactions with peptides arising from gastric and/or duodenal digestion.

The degree of protection varied depending on the polysaccharide, due to their different chemical structures and physicochemical properties. The highest resistance to digestion was found in protein and P mixtures. It is well known that P is prone to form gels at low pH, which could hinder digestion by increasing the viscosity of the gastric environment although its capacity is reduced at higher pHs like in the intestine (Polovic, Blanusa, Gavrovic-Jankulovic, Atanaskovic-Markovic, Burazer, Jankov, et al., 2007) although it has been reported that the P gels could resist enough at duodenal level to exert a protector effect in the allergen Act c 1 from kiwi (Polovic et al., 2009). Unlike previous results obtained in mixtures of polysaccharides and β-lactoglobulin (Mouecoucou et al., 2004b), in our study, X exerted the least remarkable effect.

3.2 IgE-binding of the mixtures of OVA and OM with polysaccharides

The IgE-binding of OVA, OM and the three polysaccharides studied was assessed by inhibition ELISA, what showed that the polysaccharides did not bind IgE from egg allergic patients (results not shown). However, as shown in Figures 5 and 6, the duodenal digests of OVA and OM in the presence of P, G and X had lower EC_{50} than the duodenal digests of the isolated proteins. This higher binding activity to IgE could be, at least partially, attributed to the lower digestion degree of the proteins in the presence of the polysaccharides. However, it was noteworthy that, in the presence of the

polysaccharides, the reactivity towards IgE was significantly reduced, while the digestibility of both proteins, and in particular that of OM, was only slightly affected (Figures 1 and 2), suggesting that there could be another explanation for the increased immunoreactivity observed.

To assess this point, the immunoreactivity of the compounds attributed to the interaction of OM duodenal degradation products wit hteh polysaccharides was estimated. For this purpose, the peaks eluting before 5 and 6 min in the SEC analyses of the duodenal digests of OM in the presence of P, G or X (marked with * or ** in Figure 4) were collected, and their IgE-binding activity was checked by indirect ELISA (Figure 7). The three collected fractions showed the ability to recognized IgE from egg allergic patients, with those formed in the presence of X being the most reactive in terms of IgE-binding. Thus, the presence of allergenic fragments bound to the polysaccharides could affect the IgE-binding ability of the duodenal digests. It has been found that the interaction of polysaccharides with gastric and duodenal digests reduce the IgE-binding, what was attributed to a masking effect of nthe reactive epitopes(Mouecoucou et al., 2007). In particular, X was reported to be prone to react with small peptides producing a decrease in the immunoreactivity (Mouecoucou et al., 2004a). The ability of X to interact with small protein fragments was also found in our study, however, it is reasonable that depending on the nature of the digested allergens, their interactions with X result either masking of epitopes, but also enhacing its contact to the IgE.

In conclusion, the present study shows that the presence of P, G and X, hampered the digestibility of the main allergens, OVA and OM. Changes in the digestibility of OVA and OM in the presence of polysaccharides resulted in duodenal digests that reatined higher IgE-binding of some of the hydrolysis products that only arose in the presence of polysaccharides and could result from the interaction between

the polysaccharides and the peptides derived from protein digesion. Overall, the present results underline the importance of the food matrix in the digestibility of food allergens and in their potential ability to trigger an immunse response

Abbreviations used

OVA: ovalbumin; OM: ovomucoid; P: pectin; G: gum arabic; X: xylan; GD: gastric digest; DD: duodenal digest.

Acknowledgments

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Figure captions

Figure 1. RP-HPLC analyses of duodenal digests (DD) of OVA and OVA after duodenal digestion in the presence of pectin (P), gum arabic (G) and xylan (X).

Figure 2. RP-HPLC analyses of duoodenal digests (DD) of OM and OM after duodenal digestion in the presence of pectin (P), gum arabic (G) and xylan (X) (c).

Figure 3. SEC profiles of non-digested OVA blended with pectin (P) (a); gum arabic (G) (b); and xylan (X) (c) and their respective gastric (GD) and duodenal digests (DD).

Figure 4. SEC profiles of non-digested OM blended with pectin (P) (a); gum arabic (G) (b); and xylan (X) (c) and their respective gastric (GD) and duodenal digests (DD).

Figure 5. Binding to human IgE of the duodenal digests (DD) of OVA and DD of OVA blended with pectin (P), gum arabic (G) and xylan (X) tested with 3 pool of egg allergic patient by inhibition ELISA.

Figure 6. Binding to human IgE of the duodenal digests (DD) of OM and DD of OM blended with pectin (P), gum arabic (G) and xylan (X) tested with 3 pool of egg allergic patient by inhibition ELISA.

Figure 7. Binding to human IgE of the initial fractions collected by SEC of the duodenal digests (DD) of OM and OM blended with pectin (P), gum arabic (G) and xylan (X) tested with three pool of egg allergic patient by indirect ELISA; C+: positive control.

Tables

Table 1. Specific IgE levels (kU/L) towards egg, egg white, OVA and OM of the sera used in the study.

Serum	Patient	Egg	Egg white	OVA	OM
Pool 1	1	-	10.2	11.1	2.94
	2	-	>100	78,9	69,2
	3	-	-	15	16
Pool 2	4	-	7	7,84	1,4
	5	>100	-	-	-
	6	>100	-	-	-
Pool 3	7	-	7,44	3,03	7,51
	8	>100	-	-	-
	9	-	-	62	80

Figures

Figure 1

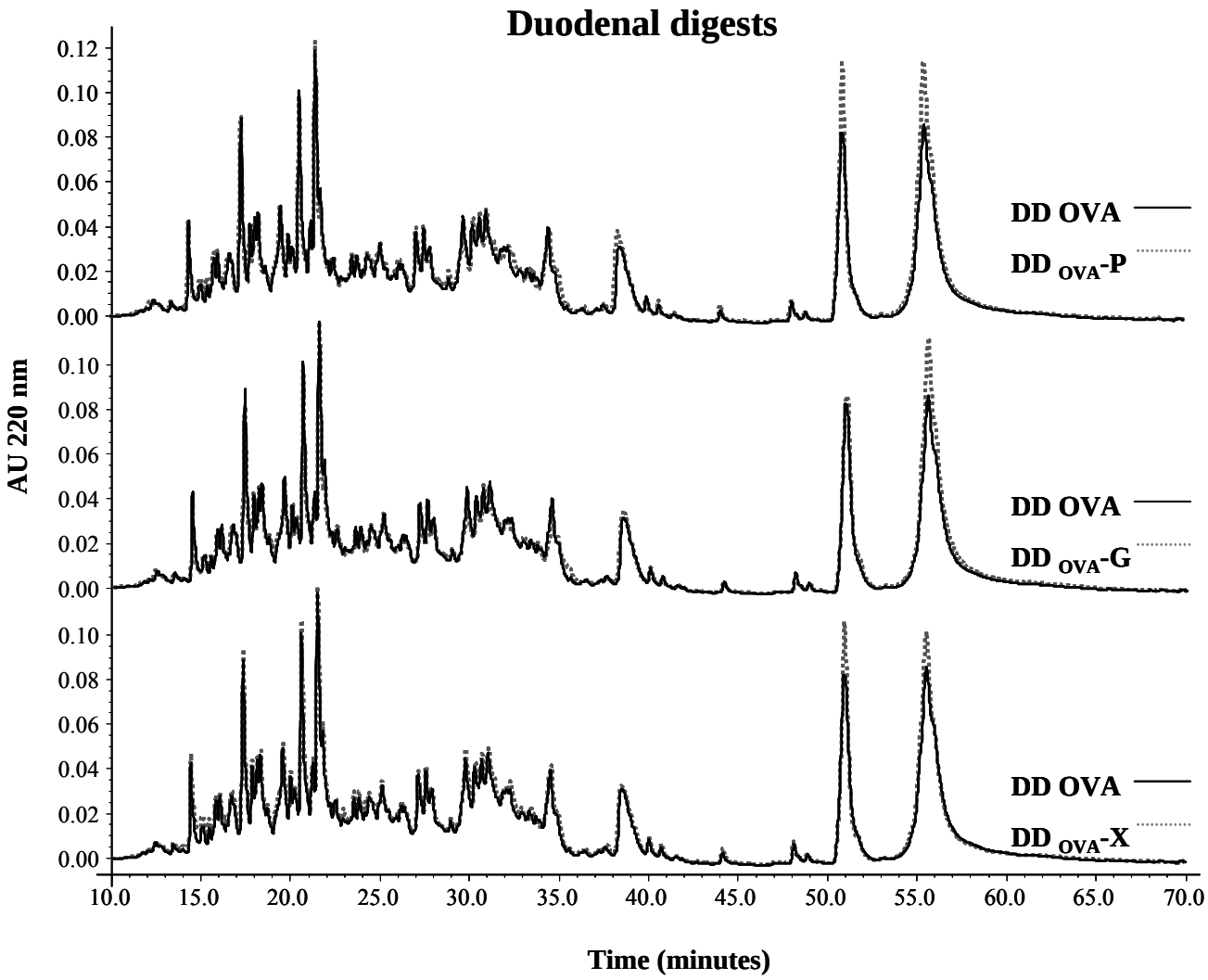


Figure 2

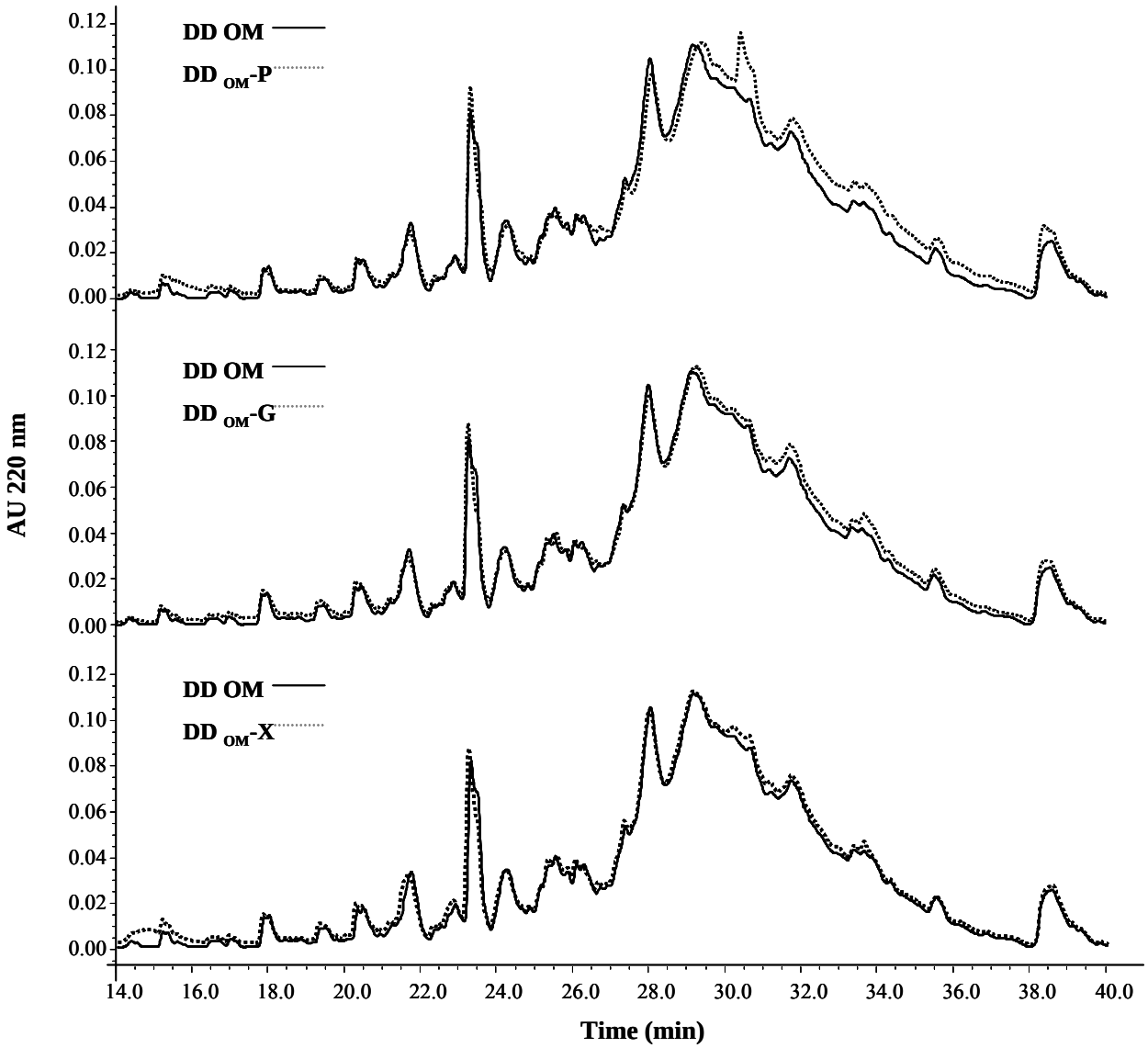

DD OM
DD OM-P
DD OM
DD OM-G
DD OM
DD OM-X
AU 220 nm
Time (min)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
14.0
16.0
18.0
20.0
22.0
24.0
26.0
28.0
30.0
32.0
34.0
36.0
38.0
40.0

Figure 3

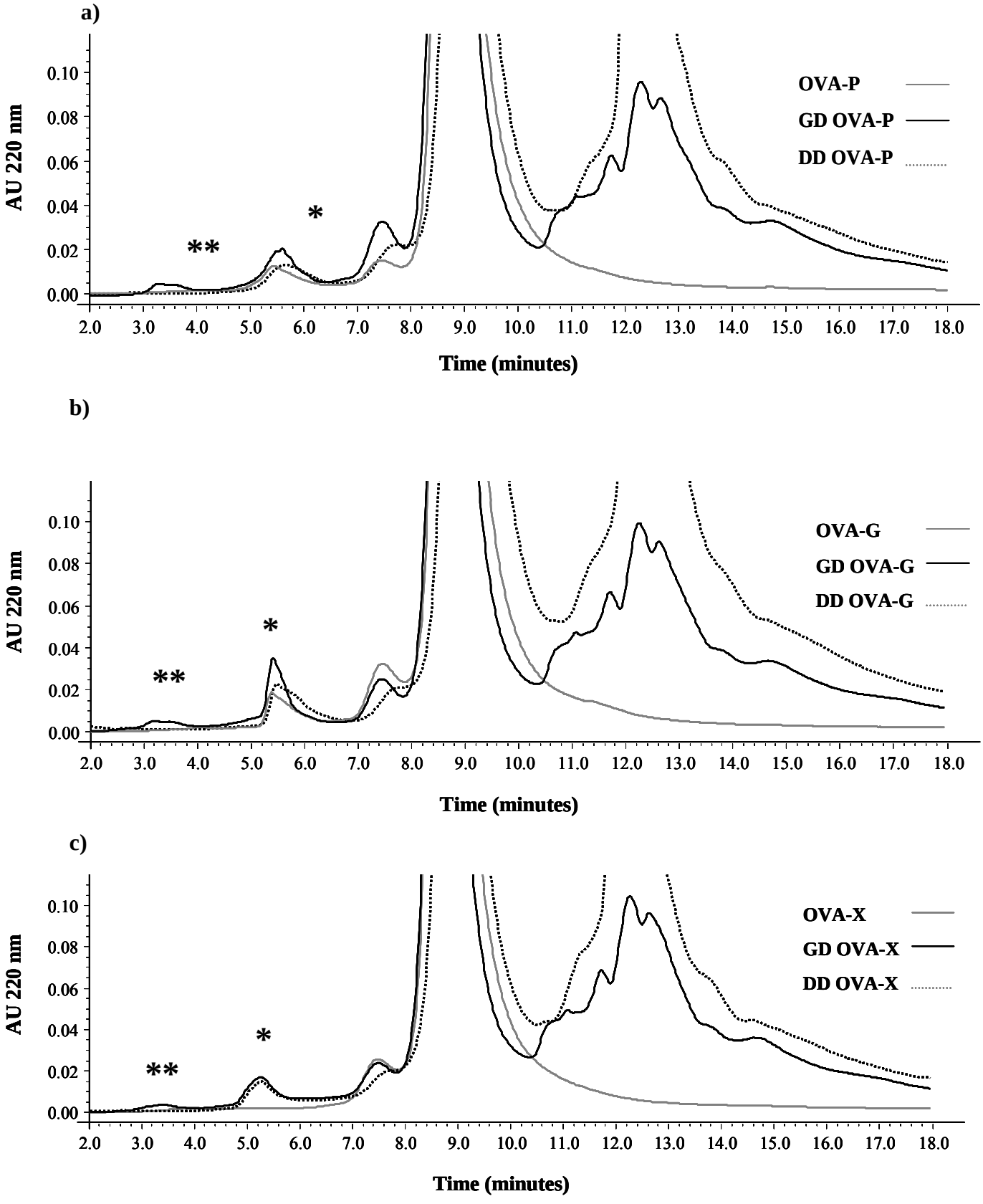
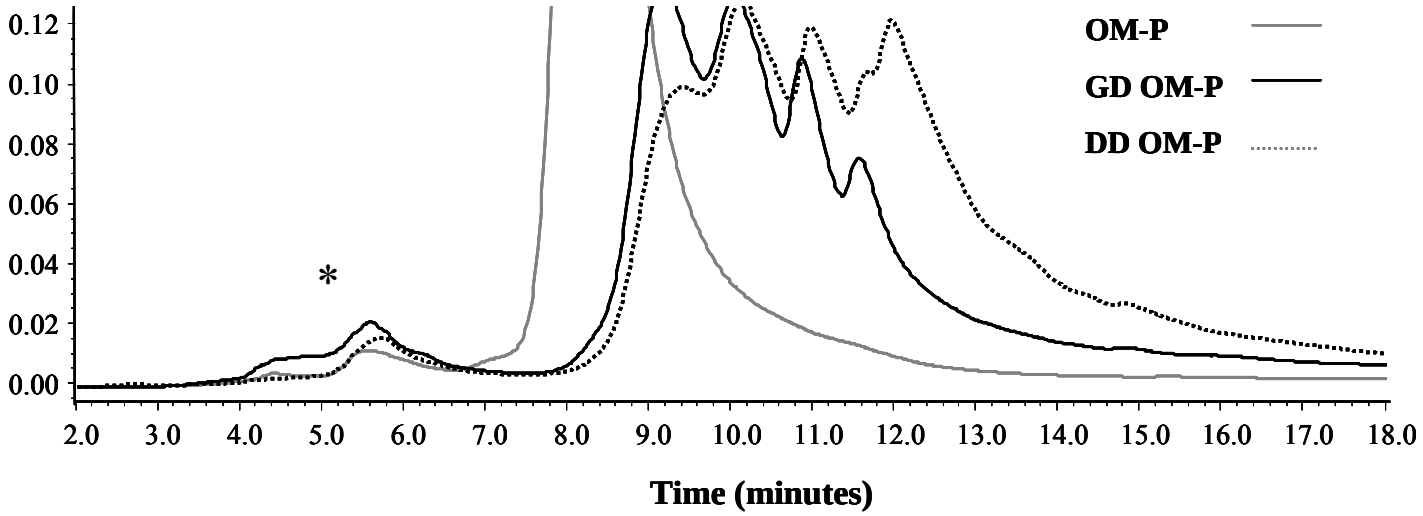
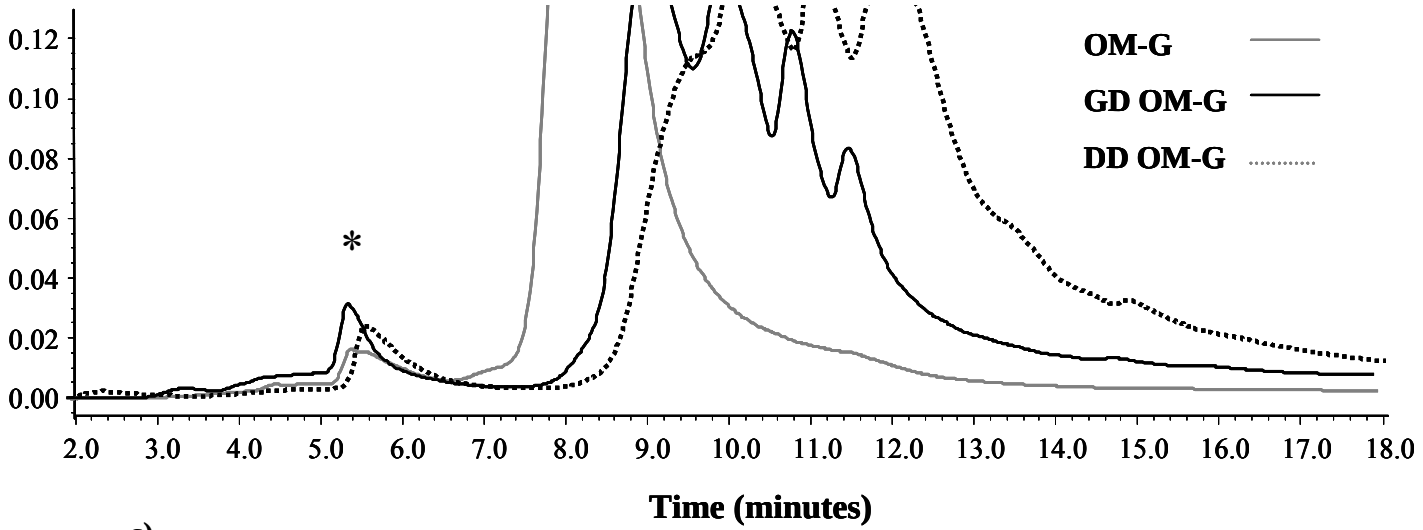

a)
AU 220 nm
0.10
0.08
0.06
0.04
0.02
0.00
**
*
OVA-P
GD OVA-P
DD OVA-P
2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0 16.0 17.0 18.0
Time (minutes)
b)
AU 220 nm
0.10
0.08
0.06
0.04
0.02
0.00
**
*
OVA-G
GD OVA-G
DD OVA-G
2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0 16.0 17.0 18.0
Time (minutes)
c)
AU 220 nm
0.10
0.08
0.06
0.04
0.02
0.00
**
*
OVA-X
GD OVA-X
DD OVA-X
2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0 16.0 17.0 18.0
Time (minutes)

Figure 4

a)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
OM-P
GD OM-P
DD OM-P
2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0 16.0 17.0 18.0
Time (minutes)

b)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
OM-G
GD OM-G
DD OM-G
2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0 16.0 17.0 18.0
Time (minutes)

c)

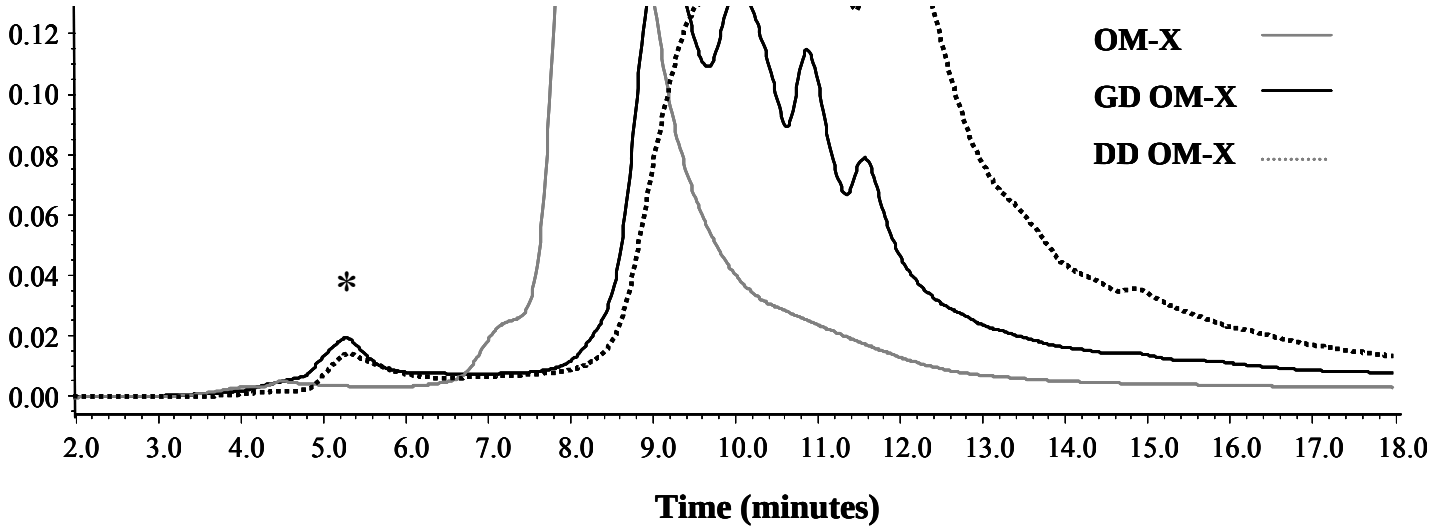

0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
OM-X
GD OM-X
DD OM-X
2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0 16.0 17.0 18.0
Time (minutes)

Figure 5

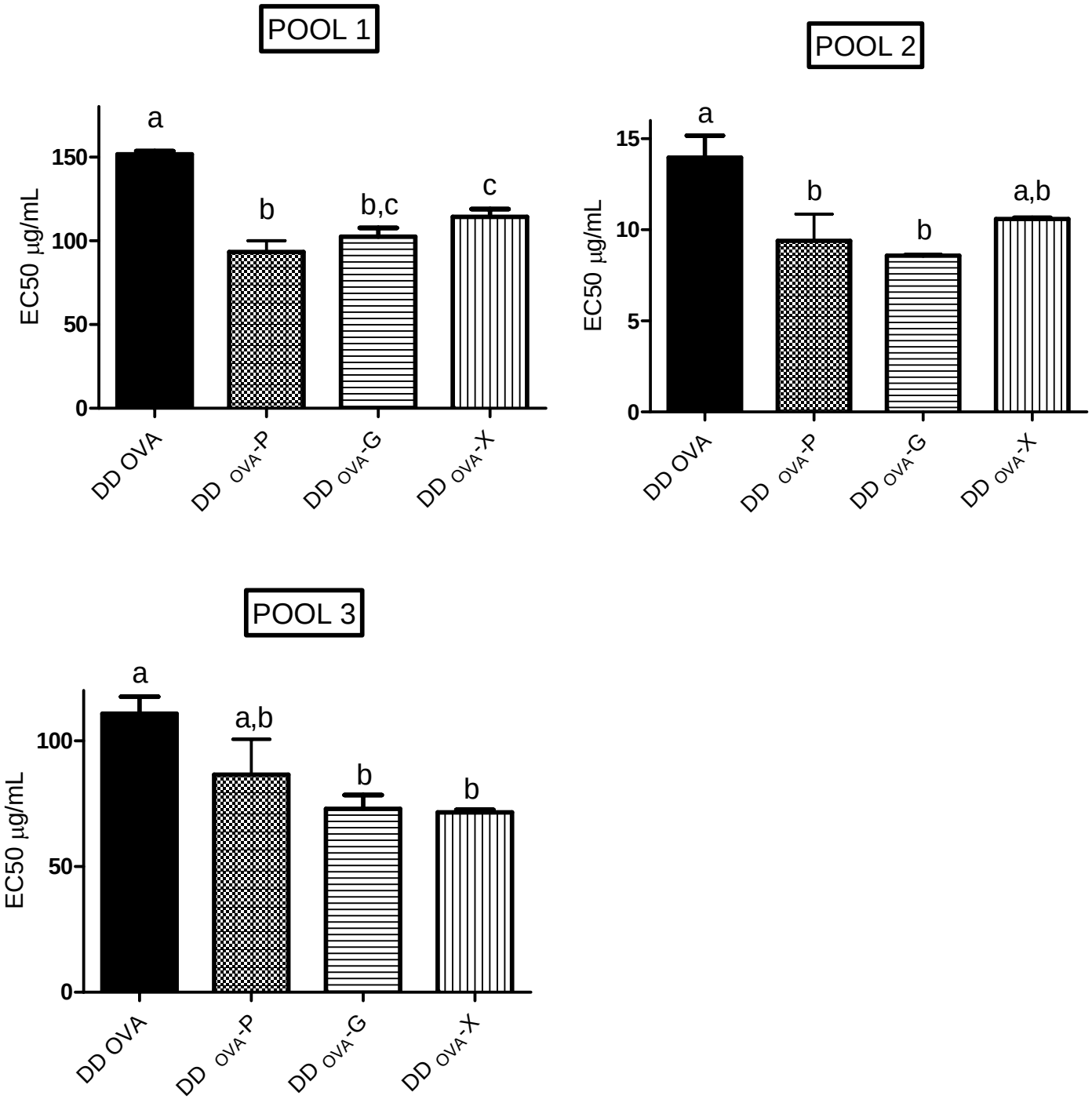

POOL 1
EC50 µg/mL
150
100
50
0
a
b
b,c
c
DD OVA
DD OVA-P
DD OVA-G
DD OVA-X
POOL 2
EC50 µg/mL
15
10
5
0
a
b
b
a,b
DD OVA
DD OVA-P
DD OVA-G
DD OVA-X
POOL 3
EC50 µg/mL
100
50
0
a
a,b
b
b
DD OVA
DD OVA-P
DD OVA-G
DD OVA-X

Figure 6

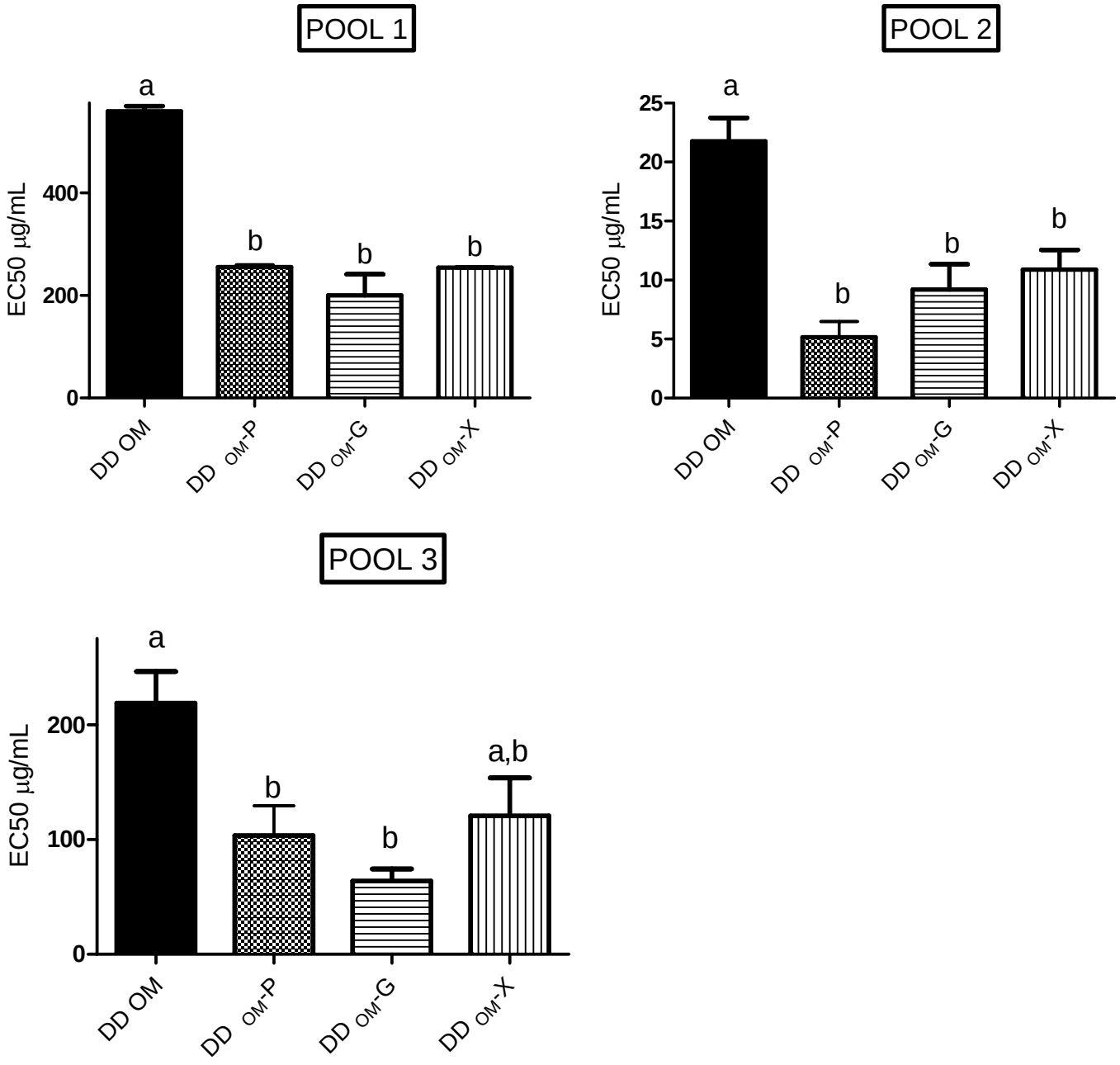
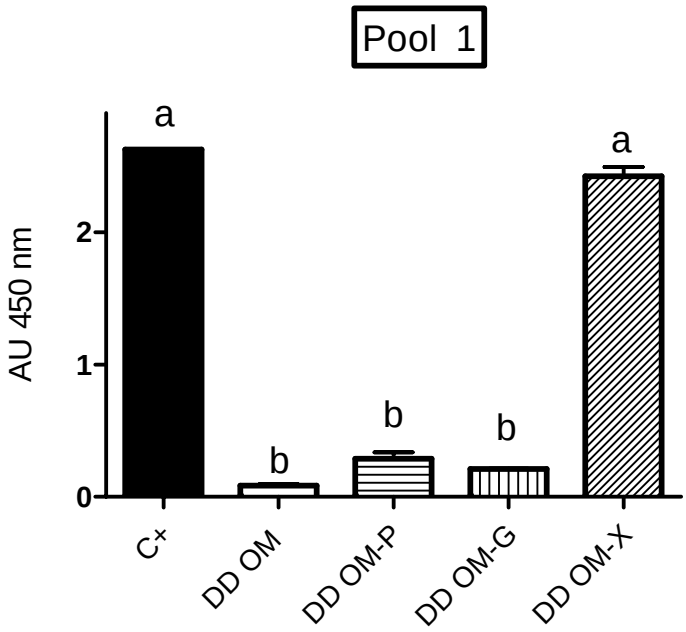
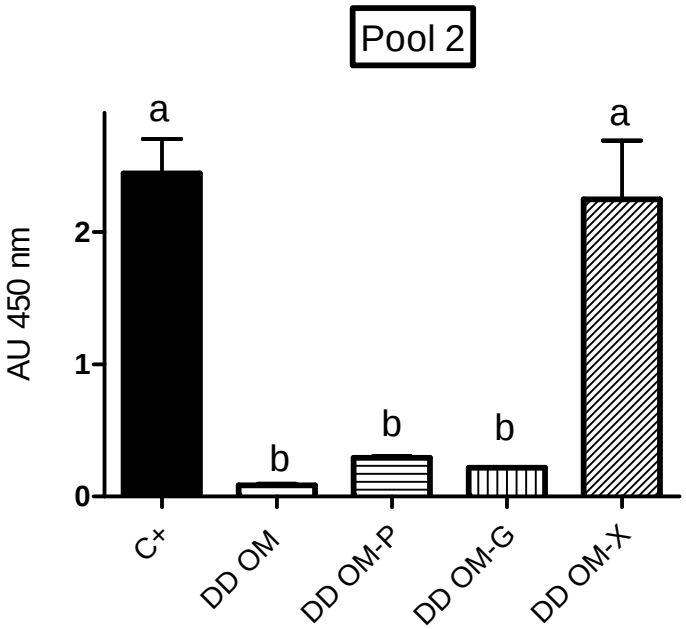
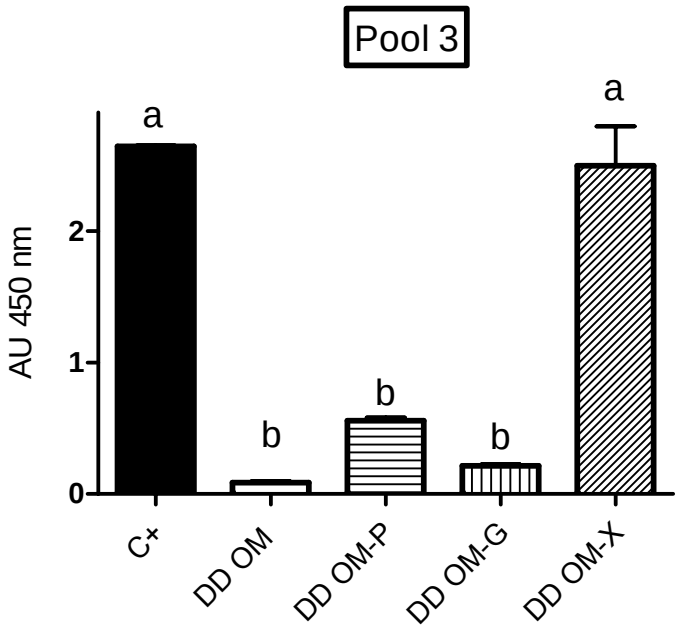

POOL 1
EC50 µg/mL
400
200
0
a
b
b
b
DD OM
DD OM-P
DD OM-G
DD OM-X
POOL 2
EC50 µg/mL
25
20
15
10
5
0
a
b
b
b
DD OM
DD OM-P
DD OM-G
DD OM-X
POOL 3
EC50 µg/mL
200
100
0
a
b
b
a,b
DD OM
DD OM-P
DD OM-G
DD OM-X

Figure 7


Pool 1
AU 450 nm
0
1
2
a
b
b
b
a
C+
DD OM
DD OM-P
DD OM-G
DD OM-X


Pool 2
AU 450 nm
0
1
2
a
b
b
b
a
C+
DD OM
DD OM-P
DD OM-G
DD OM-X


Pool 3
AU 450 nm
0
1
2
a
b
b
b
a
C+
DD OM
DD OM-P
DD OM-G
DD OM-X

Food Res. Int. (Enviado)

In vitro digestibility and allergenicity of emulsified hen egg

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Food Research International

In vitro digestibility and allergenicity of emulsified hen egg

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Abstract

Whole hen egg produced a fine stable O/W emulsion. The presence of egg proteins as part of the emulsion did not change their IgE binding, but it slightly increased the digestibility of the main allergens present in the egg-white. This indicates that, in the case of egg white proteins, there were not adsorption-induced changes that would considerably increase their flexibility and proteinase susceptibility. The increased digestibility of the emulsion resulted in a lower IgE-binding capacity of the *in vitro* gastric and duodenal digests compared to those obtained from the egg in solution.

Keywords: egg; emulsion; in vitro digestion; IgE-binding; allergenicity; food matrix.

Abbreviations: EM: egg emulsion; ES: egg solution; OVA: ovalbumin; OM: ovomucoid; LYS: lysozyme; OVT: ovotrasnferrin; GD: gastric digestion; DD: duodenal digestion; β-Lg: β-lactoglobulin, PC: phosphatidylcholine; CEM: cream phase of the egg proteins emulsion.

1. Introduction

Hen´s egg, either as a whole or its constituents (egg yolk and white), is a key ingredient in many food products. Egg proteins are extensively utilized in many foods by virtue of their nutritional value and important functional properties for industrial applications. These include gel formation, foaming capacity and emulsifying ability, among others, that are useful in bakery foods, bakery mixes, mayonnaise, salad dressing, and many convenience foods (Campbell, Raikos, & Euston, 2003). Nevertheless, egg is also known because of its allergenic potential and, in fact, it is regarded as one of the most allergenic foods. Egg allergens have been studied in depth. The major ones: ovalbumin (OVA), ovomucoid (OM), lysozyme (LYS) and ovotransferrin (OVT) are found in the egg white, but the egg yolk also contains allergenic proteins such as α-livetin and apovitellenins I and VI (Mine & Yang, 2008).

While the oral mucosa is, in some cases, the first place of antigen uptake, the ability of food proteins to sensitize and/or elicit allergic reactions is linked to their resistance to gastroduodenal digestion, which ultimately lets them interact with the intestinal mucosa where absorption occurs (Untersmayr & Jensen-Jarolim, 2006). Therefore, any factor that affects protein digestibility, whether increasing, decreasing it, or inducing different proteolysis pattern, might change its capacity to induce or trigger an allergic reaction. In this respect, the food matrix and the processing practices greatly influence the allergic potential of food proteins (Foegeding & Davis, 2011).

Interactions of proteins with lipids to form emulsions and other structures are deliberately introduced during the preparation of foods or may occur in the gastrointestinal tract as a consequence of the digestive process. Proteins, due to their amphipathic nature, adsorb efficiently at the oil/water interface, lowering the surface tension and stabilizing the system and, as a consequence, they may undergo

conformational changes. Thus, Agboola and Dalgleish (1996) reported that adsorbed β-lactoglobulin (β-Lg) is more easily digested by trypsin than soluble β-Lg, an observation that they attributed to a greater flexibility and accessibility of susceptible bonds on the interface. Similarly, the rate of pepsin digestion of β-Lg and β-casein is increased when they are presented in emulsions (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Sarkar, Goh, Singh, & Singh, 2009). On the other hand, recent work has highlighted the importance of physiological surfactants, such as phospholipids, bile salts and lipolysis products, in the gastrointestinal processing of proteins adsorbed to emulsions, but also in solution (Mackie & Macierzanka, 2010; Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). However, despite the increasing scientific interest on the behaviour of food-protein stabilized emulsions during simulated gastrointestinal digestion, to the best of our knowledge, there are not studies on the effect of emulsification on egg proteins, in terms of digestibility and allergenicity.

The long-term physicochemical stability of egg-stabilized emulsions largely depends on the presence of egg yolk in the system. This implies that the more flexible and surface-active yolk lipoproteins are preferred on the interfaces to the globular egg white proteins. While egg-white proteins adsorb to the interfaces covered with yolk lipoproteins only to a limited extent (Drakos & Kiosseoglou, 2008), it cannot be excluded that the adsorbed proteins as well as those present in the serum phase of the emulsion behave towards digestion differently than the native proteins in solution (Sarkar et al., 2009). From this background, this work studies the *in vitro* digestibility and human IgE-binding capacity of egg proteins that form part of food emulsions under the conditions that these products are usually consumed, with the aim to achieve a better understanding of the gastrointestinal processing of egg emulsions and the way the emulsion system affects the allergenic potential of the egg.

2. Materials and methods

2.1 Materials and emulsions processing

Fresh hen eggs (category A) and extra virgin olive oil were bought at a local store. The eggs were cracked, pooled and gently homogenized by using an Ultra-Turrax (T-25 basic, IKA, Germany), avoiding foam formation, freeze dried and stored at -20ºC. The pH of the pool was 7.46 and the protein content 53.4%, as estimated by the Kjeldahl method. The oil was purified by mixing with 10% (w/v) Florisil (Sigma-Aldrich, MO, USA;Bahtz, Knorr, Tedeschi, Leser, Valles-Pamies, & Miller, 2009) with vigorous stirring for 48 hours at room temperature (RT). After another 48 h, the oil was centrifuged (5000 g) and the supernatant was removed and stored at RT avoiding light exposure until used.

Emulsions (EM) were prepared with 8% (w/v) freeze dried egg (4.27% of egg protein) and 25% of olive oil (v/v) in 0.15 M NaCl adjusted with citric acid to pH 4.2. Egg solutions (ES) were gently stirred for 30 minutes at RT and, upon addition of the oil, they were homogenized by using an Ultra-Turrax (T-25 basic) at 11.000 rpm. A total volume of 50 mL of emulsion was prepared each time. The cream phases of the EM were obtained by centrifugation (10,000 g) and careful removal of the subnatants.

2.2 Stability of the EM

2.2.1 Conductivity measurements

The specific electrical conductivity of the EM was checked, at least in triplicate, by using a conductivity meter (Konductometer CG 855, Schott, Mainz, Germany). The conductivity measurements are directly related to the creaming of the droplets (Kato, Fujishige, Matsudomi, & Kobayashi, 1985). The conductivity was checked in the ES before adding the oil and in the EM at different times, up to 240 h at RT.

2.2.2 Phase separation

Digital pictures of the EM (fresh and stored for 4 h) placed into a 15 mL graduated tube were taken at different times in order to corroborate the absence of phase separation. The tubes were kept at RT and at least duplicates of each sample were prepared. A digital camera (IXUS 100IS, Canon) was used.

2.2.3 Differential interference contrast microscopy (DIC)

The microstructure of the EM (fresh emulsions and emulsions after 4 h) was studied using a DIC microscope Leica model AF6000 LX (Wetzlar, Germany). A drop of the EM was placed on the slide, covered by a cover slip, and observed under a magnification of x20. At least 2 replications were prepared for each sample and they were examined without any previous dilution. Images of the EM were taken using a digital camera (Andor 885, Belfast, Ireland) from different fields on each slide.

2.3 In vitro gastro duodenal digestions.

In vitro digestions were performed following Moreno, Mellon, Wickham, Bottrill and Mills (2005b) and Martos, Contreras, Molina and Lopez-Fandiño (2010). ES and EM were subjected to gastric digestion and aliquots were taken at 10, 20, 30 and 60 min. 60 min-gastric digests were readjusted at pH 6.5 and subjected to duodenal digestions. Duodenal digestions were stopped after 30 min with Orlistat (O4139, Sigma-Aldrich, enzyme/substrate ratio 1:70 w/w) and Bowman-Birk trypsin-chymotrypsin inhibitor from soybean (T9777, Sigma-Aldrich). Aliquots were kept at -20ºC until use for SDS-PAGE, RP-HPLC and ELISA analysis.

2.4 SDS-PAGE

SDS–PAGE was performed using Criterion XT Precast Gels with 4-12% Bis-Tris (Bio-Rad Laboratories, Hercules, CA). Samples were diluted in Laemmli sample buffer and heated at 95 ºC for 10 min. Electrophoresis was carried out at 90-100 V for 2 h and at RT, in XT MES running buffer (Bio-Rad). Gels were fixed in a 40% methanol and 10% (wt/v) acetic acid solution, followed by staining with Coomassie Blue R-250 (Bio-Rad). The precision plus protein unstained standards (Bio-Rad) containing were used. Images were taken by using a Versa Doc imaging system (Bio-Rad).

2.5 RP-HPLC

Analyses were conducted in a Hi-Pore RP-318 (250 x 4.6 mm internal diameter) column (Waters, Milford, MA) in a Waters 600 HPLC system. Solvent A was 0.37% (v/v) trifluoroacetic acid (Scharlau Chemie, Barcelona, Spain) in double-distilled water and solvent B was 0.27% (v/v) trifluoroacetic acid in HPLC-grade acetonitrile (Lab-Scan, Gliwice, Poland). The chromatographic conditions were as in Quiros, Chichon, Recio and Lopez-Fandiño (2007). Detection was at 220 nm and data were processed by using Empower 2 Software (Waters).

2.6 Human IgE binding by inhibition ELISA

Human-IgE binding was evaluated by inhibition ELISA following Jimenez-Saiz, Martos, Carrillo, Lopez-Fandiño, and Molina (2011b) with some variations: freeze dried egg was used as a coating antigen diluted in 0.1 M bicarbonate buffer, pH 9.6 to 200µg/mL; a total of 9 sera from egg allergic patients were mixed in three different pools (Table 1). Polyclonal rabbit anti-human IgE (A0094, Dako, Glostrup, Denmark) and polyclonal swine anti-rabbit immunoglobulins labelled with horseradish peroxidase (P0399, Dako) were used diluted 1:1000 and 1:2000 (v/v) respectively, in phosphate buffer, pH 7.4, containing 0.05% Tween 20 were used.

IgE-binding results were statistically processed. A non-linear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoid curve of inhibition dose-response with variable slope, from which the IC_{50} (the protein concentration that binds 50% of seric IgE) was obtained with the program GraphPad Prism 5 for Windows (GraphPad software, San Diego, CA). The IC_{50} of each sample were expressed as a mean ± standard error for n =2. Significant differences ($p < 0.05$) were evaluated by one-way analysis of variance followed by post hoc multiple-comparison using Tukey's test.

3. Results and discussion

3.1 Characterization of EM stability

Conductivity (mS/cm) is commonly used as a measurement of the stability of emulsions (Azzam & Omari, 2002; Kato, Fujishige, Matsudomi, & Kobayashi, 1985). As shown in Figure 1, the conductivity of the EM was lower than the conductivity of the ES and it remained stable during the first 48 h, showing that the oil droplets were homogeneously distributed in the entire sample volume (Gundersen, Saether, & Sjoblom, 2001). After 48 h, the values of conductivity started to increase, probably because the dispersed oil droplets began to rose and coalesce to form floating layer of oil (Gundersen et al., 2001).

Figure 2 shows the visual appearance and the microstructure of EM, freshly prepared and after 4 h. EM were very stable, without sings of phase separation for the first 4 h. Furthermore, the images taken from the optical microscope were typical of a homogeneous emulsion with small oil drops, surrounded by a well-defined interfacial film, dispersed in a continuous phase. During 4 h, there were no relevant changes in

the microstructure of the EM, which showed a low tendency to flocculate (Camino & Pilosof, 2011).

3.2 In vitro digestibility of the EM

SDS-PAGE and RP-HPLC analyses were conducted on ES, EM and their respective digests (Figures 3 and 4) to compare their *in vitro* digestibility. Both ES and EM showed a similar *in vitro* digestion pattern by SDS-PAGE, although slight differences were found. Basically, the digestion of egg proteins seemed to be slightly favoured when they formed part of the emulsions. The most abundant allergen the egg white, OVA, that was clearly seen in the gel as large band of approximately 45 kDa, is known to be very stable to pepsin and its digestion leads to a SDS-PAGE pattern with two hydrolysis fragments of ~40 and <10 kDa (Takagi, Teshima, Okunuki, & Sawada, 2003). The band corresponding to OVA underwent a more pronounced decrease in intensity with the advance of gastric digestion in EM than in ES (Figure 3, lanes 3-6, 9-12). Accordingly, the appearance of the hydrolysis fragment of ~40 kDa was more rapid during the hydrolysis of EM. The RP-HPLC analyses (Figure 4) corroborated this observation, showing that OVA, which elutes with a retention time of 56 minutes, was digested faster as part of the EM, an effect that was still noticeable at the end of duodenal digestion (Figure 4d). Similarly, LYS, also recognized by its high stability to gastric digestion (Jimenez-Saiz et al., 2011b), was more resistant to hydrolysis by pepsin in ES than in EM (Figures 3 and 4b). On the contrary, OM is very prone to pepsin digestion, disappearing in less than 10 min, with the formation of degradation products of ~18 kDa, ~13 kDa and <3 kDa (Jimenez-Saiz, Belloque, Molina, & Lopez-Fandiño, 2011a). Under our conditions, we could not detect differences in the hydrolysis of OM between ES and EM.

An enhanced *in vitro* digestibility has been described for food proteins adsorbed on oil/water interfaces (Macierzanka et al., 2009). The emulsifying activity of proteins involves the unfolding and spreading of protein molecules at the oil water/interface, mainly through hydrophobic interactions, that can lead to changes in secondary and tertiary structure and to the exposure of buried residues with the subsequent augment of hydrolysis (Bergenstahl & Claesson, 1977; Beverung, Radke, & Blanch, 1999; Nilsson, Osmark, Fernandez, & Bergenstahl, 2007). However, due to its compact globular structure OVA exhibits poor emulsifying properties, although the egg is rich in other surface-active compounds, such as the phospholipids of the egg-yolk, which further promote interfacial adsorption of OVA (Mine, Kobayashi, Chiba, & Tada, 1992). Similarly, LYS does not exhibit good emulsifying properties, which are improved by chemical and enzymatic modifications aimed to increase the amphipathicity of the protein (Shu, Sahara, Nakamura, & Kato, 1996). An indication of the presence of egg white proteins on the interface was obtained when the emulsion was centrifuged and the cream, separated from the liquid subnatant, was analyzed by SDS-PAGE, as illustrated in Figure 5, showing the presence of the main egg white proteins and yolk proteins surrounding fat globules.

It should be noted that, in the case of other proteins, such as β-casein and β-Lg, the emulsified proteins were digested by pepsin much faster than the proteins in solution (Macierzanka et al., 2009). A significantly higher extent of hydrolysis has also been reported for unadsorbed proteins in emulsions, as compared with the proteins in solution (Nik, Wright, & Corredig, 2010; Sarkar et al., 2009). This indicates that, in the case of egg white proteins, there were not adsorption-induced changes that would considerably increase their flexibility and pepsin susceptibility, probably because those that are most resistant to pepsin action, OVA and LYS, exhibit a very high

conformational stability at pH 2.0 (de Laureto, Frare, Gottardo, van Dael, & Fontana, 2002; Tatsumi, Yoshimatsu, & Hirose, 1999). On the other hand, the simulated gastric digestion of proteins in emulsions in the presence of vesicular phosphatidylcholine (PC), as it is the case of the present study, has been reported to occur mainly in solution, because PC causes displacement of the adsorbed proteins from the interfaces (Macierzanka et al., 2009). Furthermore, PC itself affects the kinetics of pepsinolysis of certain proteins in solution such as α-lactalbumin and β-Lg (Moreno, Mackie, & Mills, 2005a). However, the presence of PC does not affect the secondary or tertiary structure of OVA and LYS in solution and it does not change the susceptibility of OVA to pepsin action, while it slightly increases LYS resistance to digestion (Jimenez-Saiz et al., 2011b; Martos et al., 2010).

In the duodenal phase of the *in vitro* digestion a destabilization of the pepsin-digested EM was visually observed (results not shown). Bile acids present in the duodenal medium easily displace proteins adsorbed at the interfaces forming mixed micelles with fatty acids, and phospholipids (Macierzanka et al., 2009; Sarkar, Horne, & Singh, 2010). Therefore, the hydrolysis would mainly occur in solution, where bile acids are found to promote digestion of several dietary proteins, as is the case of OVA or myoglobin, an effect that can be enhanced by the presence of PC (Gass, Vora, Hofmann, Gray, & Khosla, 2007; Martos et al., 2010).

3.3 Human IgE-binding of the gastroduodenal digests

Human IgE-binding of the ES and EM gastric (60 min) and duodenal digests (30 min) was estimated by inhibition ELISA as illustrated in Figure 6. The presence of egg proteins as part of the emulsion did not change their IgE binding, with both EM and ES showing similar $IC_{50.}$ The progress of digestion led to the breakdown of allergenic determinants as reflected by the higher IC_{50} of the hydrolysates. The fact that

egg protein digestibility was increased in the EM was also reflected in the IgE-binding, leading to a lower IgE-binding ability in the EM digests as compared to the ES digests, although the results varied depending on the serum pool used, reflecting different sensitivities among individuals.

As far as we know, there are no previous reports on the reactivity against IgE of emulsified proteins or their digestion products. The present results suggest that the incorporation of whole egg into an emulsion may not vary substantially the potential of egg allergens to induce adverse reactions in sensitized individuals, except for a reduced immnunoreactivity that could derive from a slightly increased susceptibility to digestion.

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Figure captions

Figure 1. Changes in conductivity of the EM with time. Open squares represent the conductivity of the EM and the close circle represents the conductivity of the ES. The inset represents the conductivity changes within the first 10 hours.

Figure 2. Schematic representation of macroscopic appearance evolution of an egg based oil-in-water emulsion with time (emulsion freshly made and after 4 h). Microscopic images were captured with zoom: 20x. The scale bar represents 20 μm.

Figure 3. SDS-PAGE analysis of gastric (GD) and duodenal (DD) digestion of egg in solution (ES) (lines 2-7) and in emulsion (EM) (lines 8-13). Line 1 represents the MW standard. OVA: ovalbumin; OVT: ovotransferrin; OM: ovomucoid; LYS: lysozyme; PC: phosphatidylcholine.

Figure 4. RP-HPLC analysis of egg proteins digestion products in solution (ES) and in emulsion (EM) using a Wi Pore C18 RP 318 (Bio-Rad, Richmond, USA) column (250 x 4.6 mm). a) substrates; b) egg proteins subjected to gastric digestion for 20 min c) egg proteins subjected to gastric digestion for 60 min; d) egg proteins subjected to gastric digestion for 60 min followed by duodenal digestion for 30 min; OVA: ovalbumin; OVT: ovotransferrin; OM: ovomucoid; LYS: lysozyme; bile salts are represented as ***.**

Figure 5. SDS-PAGE analysis of egg proteins in solution (ES) and of the cream phase of the egg proteins emulsion (CEM). Line 1 represents the MW standard; Line 2 corresponds to the ES and Line 3 to the cream of the EM.

Figure 6. Human IgE-binding by inhibition ELISA of egg proteins in solution (ES), in emulsion (EM) and their respective gastric (60 min) and duodenal (30 min) digest.

Tables

Table 1. Specific IgE levels (kU/L) towards egg, egg white, yolk, ovalbumin (OVA) and ovomucoid (OM) of the sera used in the study and age of the patients.

Serum	Patient	Age	IgE (kU/L)			
			Egg white	Egg yolk	OVA	OM
Pool 1 F	1	3	10,2	1,71	11,1	2,94
	2	12	>100	85,7	78,9	69,2
	3	-	-	-	15	16
Pool 2	4	2	7	2,2	7,84	1,4
	5	-	Egg>100			
	6	-	Egg>100			
Pool 3	7	1	7,44	0,89	3,03	7,51
	8	-	Egg>100			
	9	-	-	-	62	80

Figures

Figure 1

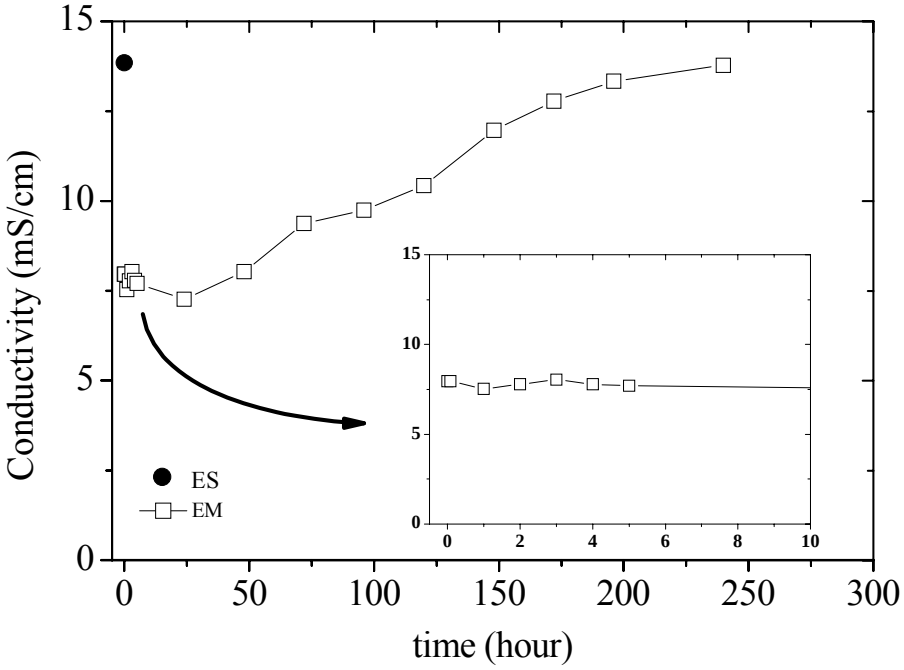

Figure 2

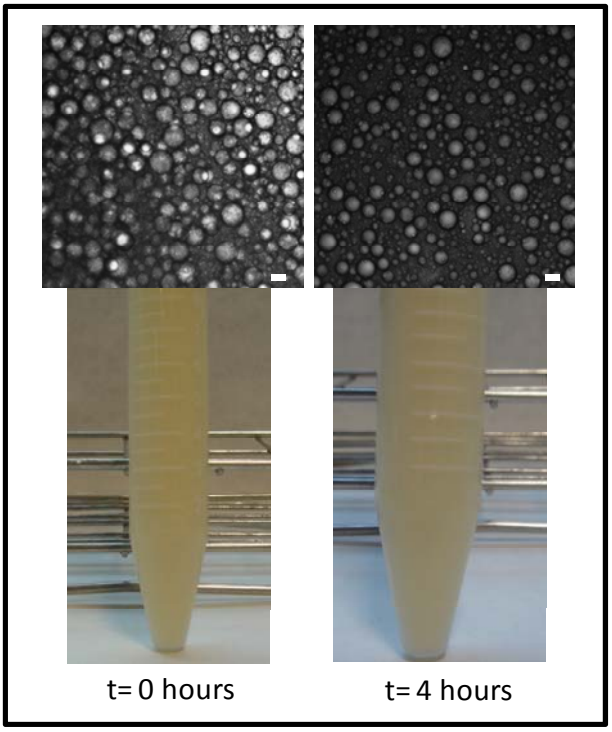

Figure 3

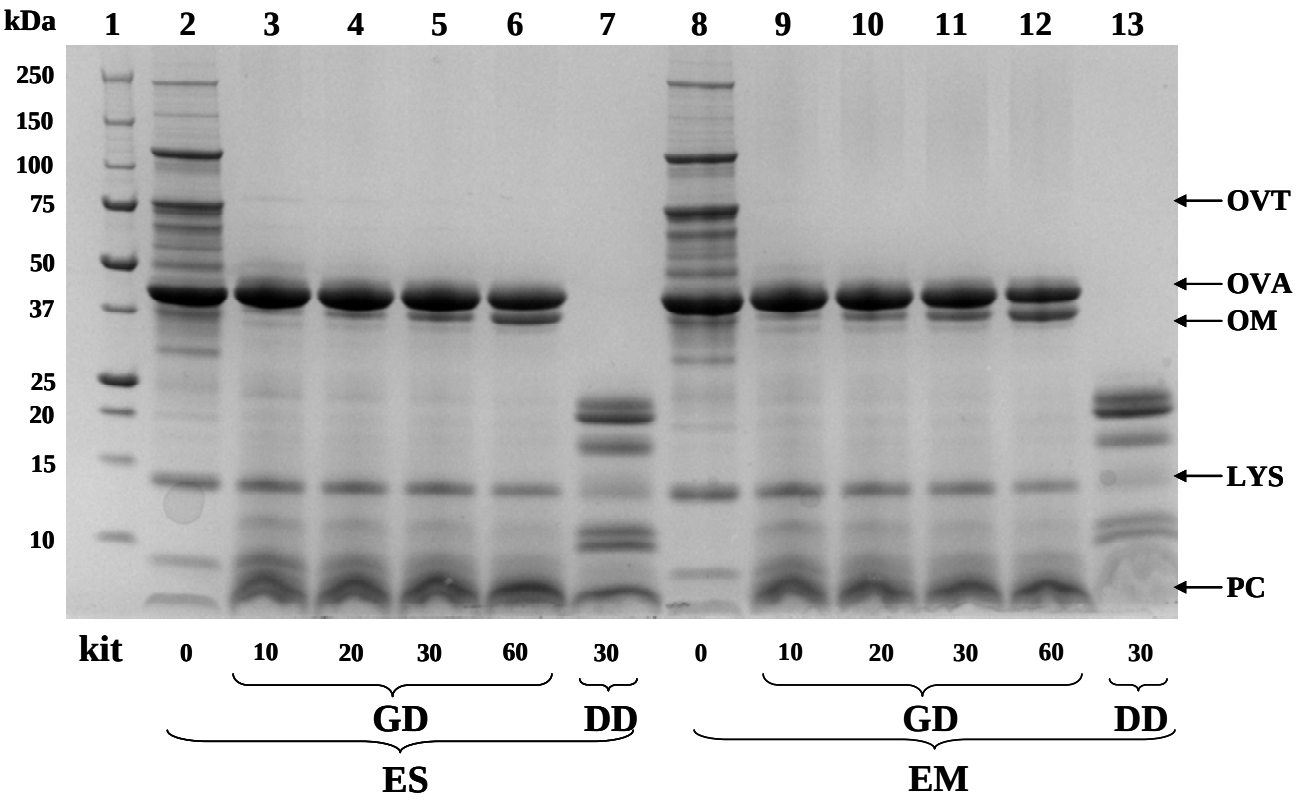
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Figure 4

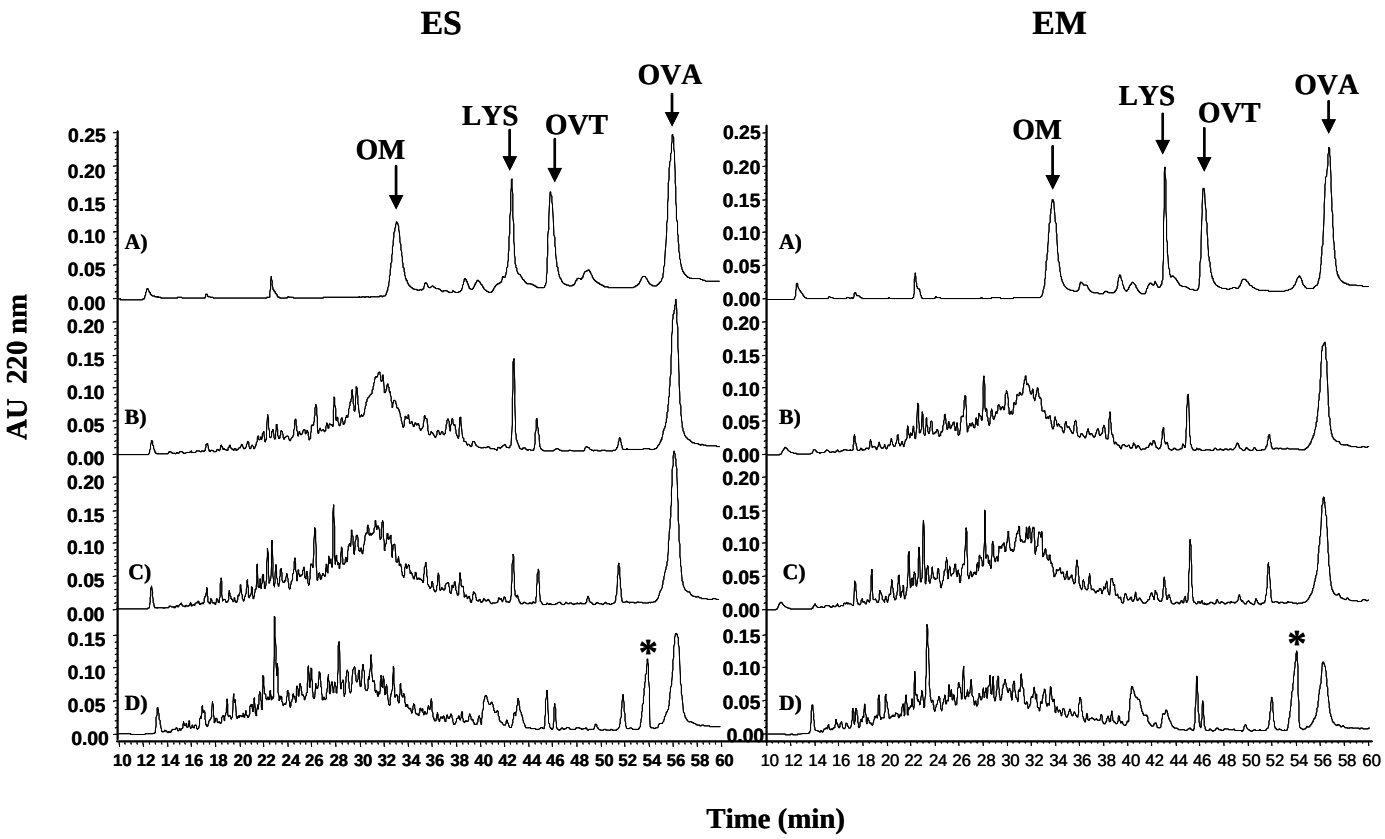


Figure 5

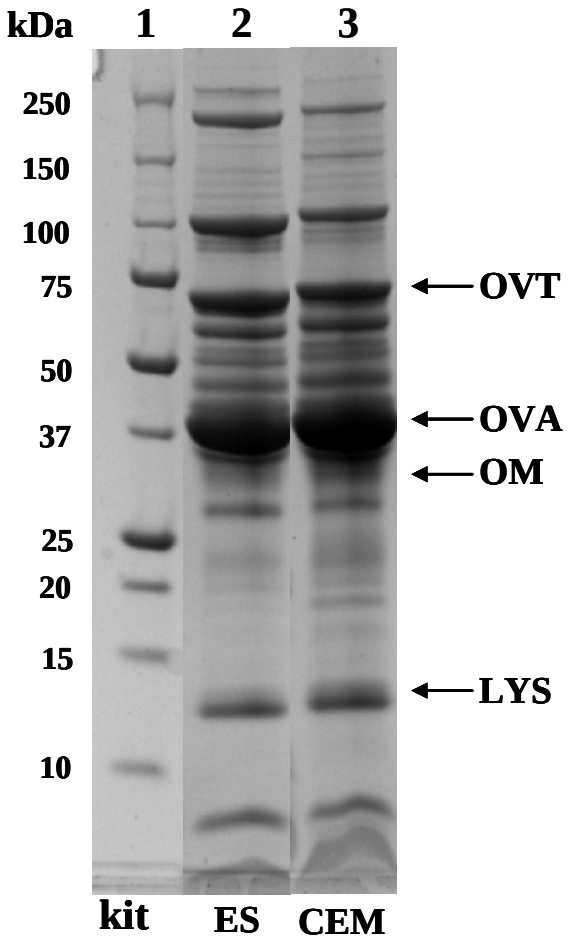
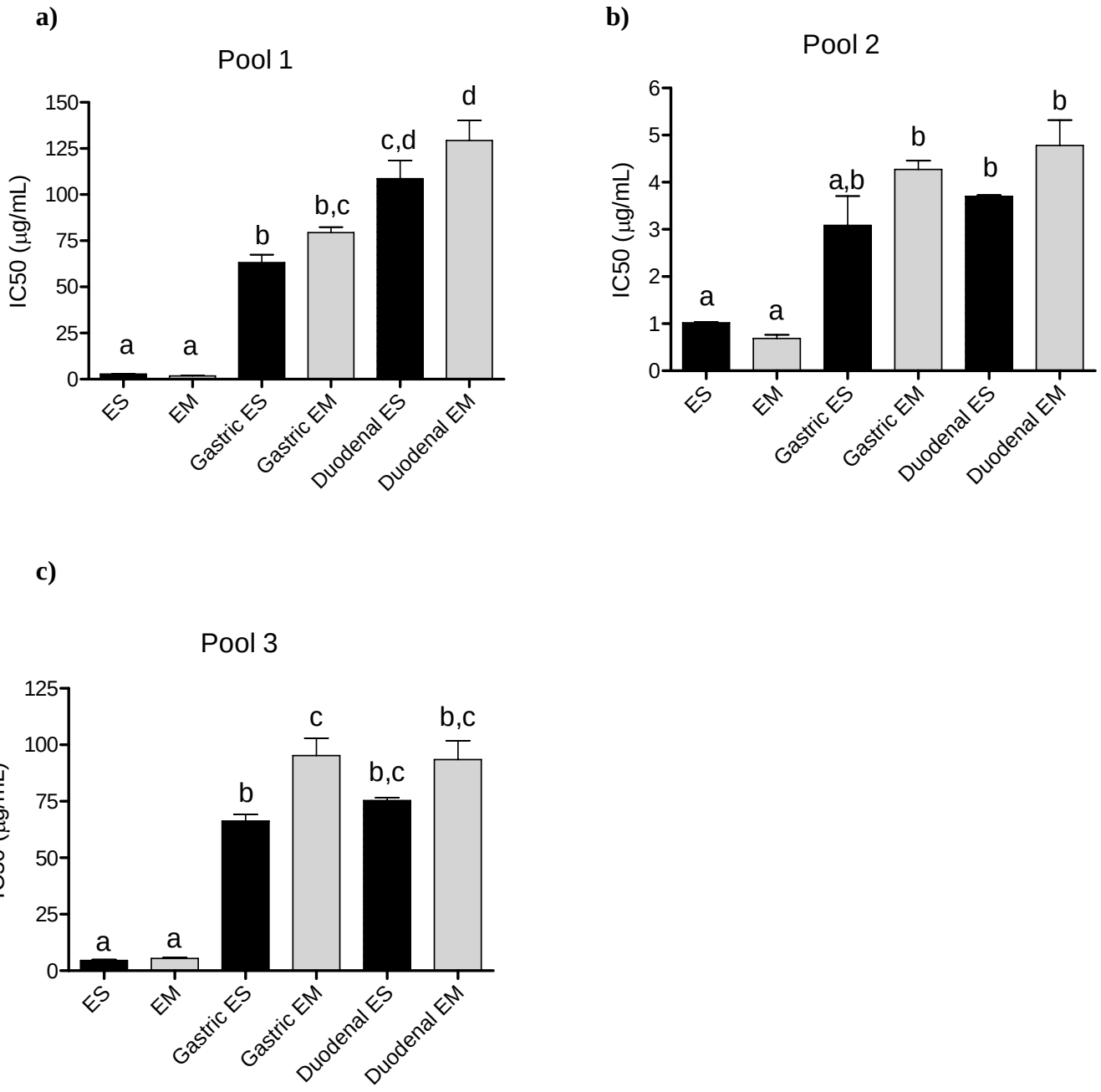


Figure 6


a)
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b
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EM
Gastric ES
Gastric EM
Duodenal ES
Duodenal EM
b)
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IC50 (µg/mL)
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2
1
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ES
EM
Gastric ES
Gastric EM
Duodenal ES
Duodenal EM
c)
Pool 3
IC50 (µg/mL)
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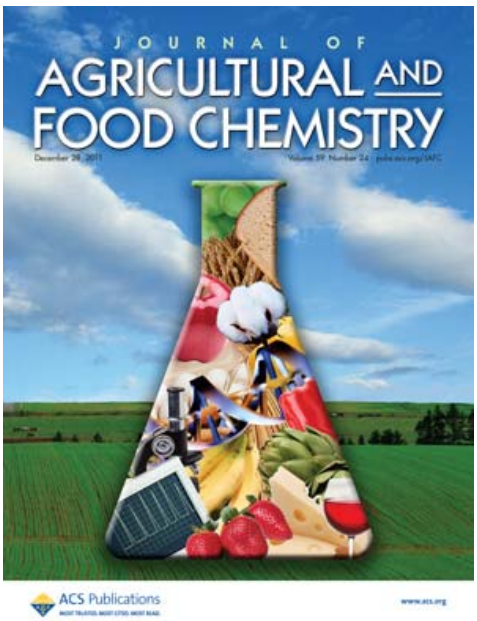

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Immunomodulatory effects of heated ovomucoid-depleted egg white in a Balb/c mouse model of egg allergy

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Immunomodulatory Effects of Heated Ovomucoid-Depleted Egg White in a BALB/c Mouse Model of Egg Allergy

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ABSTRACT: Oral immunotherapy (OIT) is a promising therapeutic approach for treating food allergy. The treatment with heated ovomucoid-depleted egg white (HOMEW) in egg-allergic patients is noteworthy; however, OIT protocols are still experimental, and a better knowledge of the underlying mechanism is required. The objective of this work was to investigate the immunomodulatory effects of HOMEW and characterize the underlying mechanism in a BALB/c mouse model of egg allergy. Mice were sensitized with EW and treated with HOMEW. Post treatment, mice were challenged with EW and euthanized for collecting blood and spleen. Markers of allergic clinical outcomes were measured as histamine concentration, serum antibody activity, and cytokine production from cultured splenocytes. Digestibility of HOMEW was assessed mimicking physiological conditions in vitro. The HOMEW demonstrated high digestibility. The treatment induced a marked increase of the Th1/Th2 ratio in the high-dose treatment group. Treated mice had significantly less histamine, EW-specific IgE, and IL-4 and more IFN-γ and IL-10. This study confirms the mechanisms involved in successful tolerance induction with OIT using HOMEW and allows understanding of the vital role of surrogate allergy markers involved in immune modulation.

KEYWORDS: food allergy, egg white, ovomucoid, BALB/c mice, oral immunotherapy

INTRODUCTION

Food allergies are recognized as a global medical problem that affects >25% of the population in industrialized countries[1] and accounts for one-third to a half of anaphylaxis cases worldwide.[2] Prevalence of food allergies is on the rise, and a concerning increase of 18% has been reported in the United States from 1997 to 2007.[3] Recently it has been reported that estimates are about 5% in children and 3−4% among adults.[4] Egg allergy is the second most common cause of food allergies in children,[5] and four major allergens in the egg white [ovomucoid (OM or Gal d 1), ovalbumin (OVA or Gal d 2), ovotransferrin (OVT or Gal d 3), and lysozyme (LYS or Gal d 4)] contribute to induction of allergy.[6] Among the four, OM is considered to be immunodominant[7] due to its stability to heat treatment[8] and enzymatic digestion[9] and its ability to retain IgE binding epitopes after in vitro digestion.[10]

At present, the main treatment for egg-allergic patients is based on food avoidance; however, this poses a challenge due to the omnipresence of eggs in a wide range of foods.[11] Furthermore, it has been shown that avoidance could lead to a lower reactivity threshold in human subjects.[12] For these reasons, a therapeutic approach seems to be more appropriate for the treatment of egg allergies. Oral immunotherapy (OIT) is one of the most studied therapeutic approaches, and encouraging results have been recently reported both in mice[13] and in humans.[14] Nevertheless, oral tolerance induction protocols to food allergens are ongoing and are only experimental because there are remaining questions that need to be answered prior to the exploration of OIT as a treatment module such as the identification of the severity and type of food allergy response to treatment, whether OIT leads to desensitization or oral tolerance, whether oral tolerance occurs naturally or is induced by treatment, the optimal dose and duration, the degree of protection, establishment of an adequate dose and whether a maintenance dose is necessary, etc. Hence, appropriate conditions for allergen preparation, treatment protocols, and outcomes for OIT need to be standardized. Although few common facts related to successful OIT have been described such as reduction in specific IgE activity, induction of IgG4/IgG2a, altered T-cell cytokine balance (shift of Th2 to Th1 type response), and T-cell anergy,[15] the overall mechanism is not completely understood, for example, the role of specific IgA exerted at mucosal level[16] or the controversial role of specific IgG and its respective isotype subclasses.[17]

With regard to egg white (EW) OIT, the use of heated EW formulas has always been a common choice and is frequently reported.[18] Treatment with heated ovomucoid-depleted egg white (HOMEW) is especially notable. HOMEW can be used in approximately 95% of egg-allergic patients because of its hypoallergenic character and has been demonstrated to be safe.[19] Furthermore, the effectiveness of HOMEW in EW-allergic patients has been proven recently,[18] but the underlying mechanism remains unclear. Reliable protocols for OIT are underway, and more light is being shed on the mechanisms involved in OIT.[14,20−22]

With this background, our objective was to study the immunomodulatory effect of HOMEW in a BALB/c mouse model of egg allergy to reach a better understanding of the mechanisms involved by which HOMEW desensitizes and may induce oral tolerance.

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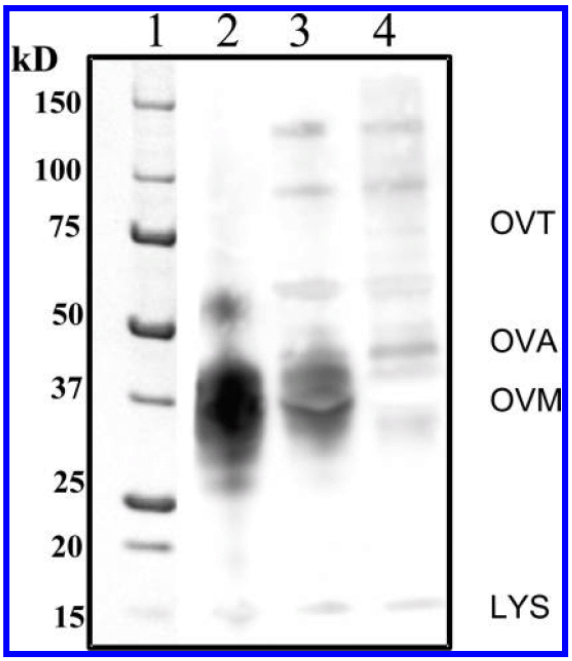

Figure 1. Immunoblot analysis of native OM, egg white (EW), and heated ovomucoid-depleted egg white (HOMEW) (20 μg/well) by using rabbit anti-OM IgG-HRP. Lanes: 1, molecular marker; 2, pure OM; 3, EW; 4, HOMEW.

MATERIALS AND METHODS

Preparation of HOMEW. HOMEW was prepared as previously described[19] with slight variations. In brief, egg white was separated and diluted (1:10, v/v) with Milli-Q water and sieved by a metallic strainer, and the pH was adjusted to 5. The sample was heated at 95 °C for 30 min. After heat treatment, the sample was centrifuged at 7000*g* at room temperature for 30 min. Because OM does not coagulate by heating, the OM was retained in the supernatant, whereas the precipitate contained the HOMEW. The precipitate was confirmed by Western blotting (Figure 1) to ensure that the sample was composed of HOMEW. The sample thus obtained was freeze-dried and stored at −30 °C for further use.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli.[23] Samples (40 μg/well) were dissolved in sample buffer in the presence of 5% (v/v) β-mercaptoethanol, heated for 5 min at 95 °C, and run on 4−12% Criterion XT gels (Bio-Rad Laboratories, Hercules, CA). Gels were stained using Coomassie G-250 (Bio-Rad) and destained using a 40% methanol−7% acetic acid solution.

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC). The RP-HPLC analysis was performed using a Waters 600 HPLC instrument (Waters, Milford, MA) and a 250 mm × 4.6 mm Widepore C18 column (Bio-Rad, Richmond, CA). Operating conditions were as follows: column at room temperature; flow rate at 1 mL/min; injection volume at 60 μL; solvent A (0.37 mL/L TFA in Milli-Q water); and solvent B (0.27 mL/L TFA in HPLC grade acetonitrile). A linear gradient of solvent B in A, from 0 to 60% in 60 min, followed by 60% B for 30 min, was used. Absorbance was recorded at 220 nm with a Waters 2487 λ dual detector. The software Empower 2000 system data (Waters) was used.

Western Blotting. Following SDS-PAGE, the proteins (20 μg/well) were transferred onto a 0.45 μm nitrocellulose membrane (Bio-Rad) using a semidry transfer cell (Bio-Rad). The membrane was blocked at room temperature for 2 h with 1% casein dissolved in 1× TBS (25 mM Tris-HCl, 0.15 M NaCl, pH 7.4; blocking buffer) and then incubated at 4 °C overnight with rabbit anti-OM IgG antibody with horseradish peroxidase (HPR) (Immune Systems Ltd., ISL, Paignton, U.K.) diluted 1:25000 in blocking buffer. Following overnight incubation, the membrane was washed (6 × 5 min) in 1× TBS with 0.05% Tween-20. Eventually the blots were visualized using the ECL prime Western blotting detection reagent (GE Heathcare, Buckinghamshire, U.K.).

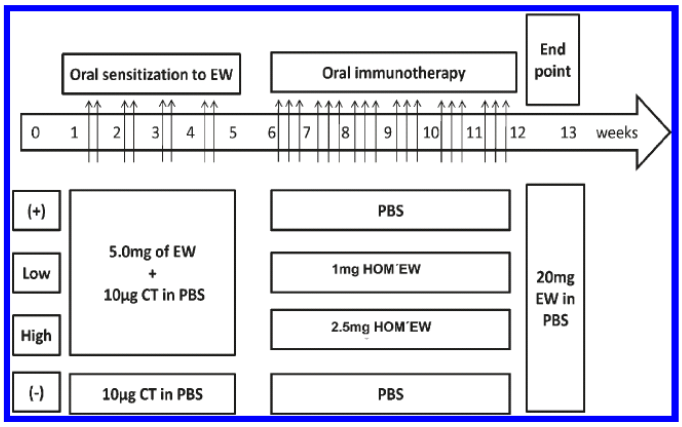

Figure 2. Groups of BALB/c mice (n = 40) were orally sensitized twice/week for 4 weeks with 5 mg of egg white and 10 μg of cholera toxin and desensitized with two different doses (1 and 2.5 mg) of heated-ovomucoid depleted egg white three times/week for 6 weeks. Mice were challenged with 20 mg of intact egg white at the end, and blood and tissue samples were collected for analysis of various parameters.

Gastric and Duodenal Digestion. Digestibility of EW and HOMEW was demonstrated using an in vitro model system in two steps, which mimics gastric and duodenal digestion in vivo.[24] Both EW and HOMEW were subjected to in vitro gastric digestion at 5.7 mg/mL final concentration. In brief, the digestions were performed in simulated gastric fluid (SGF, 35 mM NaCl) at pH 2.0, for 60 min at 37 °C, with porcine pepsin (EC 3.4.23.1, 3210 U/mg protein, Sigma-Aldrich) at an enzyme/substrate ratio (E:S) of 1:20, w/w (172 U/mg). Aliquots were taken at 0, 30, and 60 min of incubation, and adjusting the pH to 7 with 1 M $NaHCO_3$ stopped the reaction. Duodenal digestions were performed by using the 60 min gastric digests adjusted to pH 7, as described above, with the addition of 1 M $CaCl_2$, 0.25 M Bis-Tris, pH 6.5, and a 0.125 M bile salt mixture containing equimolar quantities of sodium cholate and sodium deoxycholate (Sigma-Aldrich). After incubation at 37 °C for 15 min, pancreatin (Sigma-Aldrich) was added at an enzyme/substrate ratio of 1:25, w/w. The final composition of the mixture was 4.27 mg/mL EW or HOMEW, 6.15 mM of each bile salt, 20.3 mM Bis-Tris, 7.6 mM $CaCl_2$; and pancreatin (enzyme/substrate ratio = 1:25, w/w). Aliquots were taken after 60 min of gastric digestion and 30 min of duodenal digestion. Duplicate digestions were conducted for each condition.

Animal Sensitization and Challenge. Female BALB/c mice (n = 40) were purchased from Charles River Laboratories (Montreal, QC, Canada) at 6−8 weeks of age and randomly divided into four groups (n = 10/group). All animals were housed in the campus animal facility at the University of Guelph under an egg-free diet [Teklad global diet, 14% protein (wheat and corn) and 3.5% fat] in a 12 h lighting cycle. Food and water were available ad libitum. All procedures were performed in accordance with the guidelines established by the Canadian Council of Animal Care (CCAC) and approved by the Animal Care Committee at the University of Guelph. As shown in Figure 2, following a 1 week acclimatization period, positive and treatment groups were sensitized with EW (5 mg/mouse) and 10 μg of cholera toxin (CT) (List Biologicals, Denver, CO) by oral gavage twice a week for a duration of 4 weeks. After the sensitization phase, EW-specific IgE activity was determined by ELISA to ensure that the positive and treatment groups were sensitized. After the sensitization period, HOMEW was orally

administered to the treatment groups three times a week for 6 weeks in two different doses: 2.5 mg (high-dose group) and 1.0 mg (low-dose group). The positive and negative groups received 0.01 M sodium phosphate-buffered saline solution at pH 7.4 (PBS). All mice were challenged on week 13 with 20 mg of EW diluted in PBS and euthanized for collection of blood and tissue samples.

Serum Histamine Release. Following the final oral challenge, whole blood was collected by cardiac puncture, and two serum samples were pooled in equal volumes within each group ($n = 5$) due to the limited volume in individual mouse serum for performing ELISAs. Histamine concentrations were assayed using a commercial ELISA kit (Labor Diagnostika Nord, Nordhon, Germany) following the manufacturer's instructions.

Total and Specific IgE and IgG in Serum. Concentrations of total IgE and IgG in mouse serum were determined by ELISA. In brief, flat-bottom 96-well microtiter plates (Corning, Costar Corp., MA) were coated with 100 μL of anti-mouse IgE monoclonal antibody (BD Biosciences, San Diego, CA) or goat anti-mouse IgG (Calbiochem, La Jolla, CA) at 1 μg/mL final concentration. After overnight incubation at 4 °C, the plates were washed with PBS with 0.05% (w/v) Tween-20 (PBST) three times and blocked with 200 μL of 1% (w/v) bovine serum albumin (BSA) in PBS for 2 h at 37 °C. The plates were washed with PBST three times, and dilutions of murine IgE (BD) (0.625−20 ng/mL) or IgG standards (AbD Serotec, Raleigh, NC) (0.625−40 ng/mL) and serum samples (1:50 for IgE and 1:40000 for IgG) in 1% BSA in PBST were added (100 μL) to the wells in duplicate and incubated for 2 h at 37 °C. The plates were washed further with PBST four times, and 100 μL of biotinylated anti-IgE monoclonal antibody (1:1000) (Caltag, Carlsbad, CA) or alkaline phosphate-conjugated goat anti-mouse IgG (1:2000) (Sigma-Aldrich) were added to the wells and incubated for 1 h at 37 °C. After washing four times, 100 μL of avidin−horseradish peroxidise (BD) or extravidin conjugated to alkaline phosphatase (1:3000) (Sigma-Aldrich) was added onto the plates and incubated for 30 min at 37 °C. The reaction product was visualized by adding 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich) or *p*-nitrophenol phosphatase (1 mg/mL) (Sigma-Aldrich). The reaction was incubated for 30 min followed by the addition of 25 μL of 0.5 M H_2SO_4 or 3 N NaOH (stop solution). The optical density absorbance readings (450 or 415 nm) were taken using a microplate reader (iMark Microplate reader, Bio-Rad), and readings obtained from the individual serum samples were converted to concentrations of IgE and IgG per milliliter for each assay from the values obtained from the standard curve.

Specific IgE and IgG levels were measured by coating the plates with 50 μg/mL of the intact EW, OM, or OVA, and a procedure similar to that described above was followed. Murine serum samples were diluted 1:5 for specific IgE and 1:1000 for specific IgG.

Measurement of Specific IgG1 and IgG2a Activity. Measurements of EW, OM, and OVA specific IgG1 and IgG2a in mouse serum samples were performed by an indirect ELISA. In brief, 96-well microplates (Corning) were coated with 100 μL of EW or OM (50 μg/mL) and incubated overnight at 4 °C. Plates were washed three times using PBST and blocked with 200 μL of 1% (w/v) BSA for 1 h at 37 °C. Diluted serum samples (1:10000 dilutions for specific IgG1 and 1:1000 for specific IgG2a) were added to each well and incubated for 1 h at 37 °C. After three washings with PBST, biotinylated monoclonal rat antibodies were added for IgG1 and IgG2a (100 μL/well; 1 μg/mL) (BD), and the plates were incubated for 1 h at 37 °C. The plates were washed three times and incubated further with 100 μL of avidin−HRP peroxidase-conjugated (BD) (1:2000) for 30 min at 37 °C before detection. The plate was washed six times with PBST, and the reaction was visualized using 50 μL of TMB (Sigma-Aldrich) and incubated for 30 min. The reaction was terminated by adding 25 μL of 0.5 M H_2SO_4, and absorbance was read at 450 nm using a microplate reader (iMark Microplate reader, Bio-Rad).

Measurement of EW-Specific IgA in Fecal Samples. In an effort to further elucidate the underlying mechanism occurring locally at the intestinal level, mouse fecal pellets were freshly collected on a weekly basis from each mouse group cage and were submitted to the following extraction procedure. In brief, fecal pellets were freeze-dried, diluted 1:7 (w/w) in PBS, and homogenized by using a vortex. Samples were subsequently centrifuged at 1600*g* for 15 min at 4 °C to remove large fibrous particles. Supernatants were carefully collected and centrifuged again at 9500*g* for 10 min at 4 °C. Concentrations of EW-specific IgA were determined using a sandwich ELISA procedure. In brief, flat-bottom 96-well ELISA plates (Corning) were incubated with 100 μL/well of EW (50 μg/well) in 100 mM $NaHCO_3$ (pH 9.6) and stored overnight at 4 °C. Plates were subsequently washed four times with PBST and blocked with 200 μL/well of 1% BSA in PBS and incubated for 1 h at 37 °C. An additional four washings were performed, and 100 μL/well of fecal supernatants was added onto the plate in triplicate wells and incubated overnight at 4 °C. The plates were washed four times with PBST and incubated with 100 μL/well of biotinylated monoclonal anti-mouse IgA (1: 500; BD) diluted in 1% BSA in PBST for 1 h at 37 °C. The wells were further washed four times with PBST, and 100 μL/well of avidin−HRP conjugate (1:2000; BD) was applied onto the plate for 30 min of incubation at 37 °C. After a final four-wash cycle with PBST, EW-specific IgA binding activity was revealed by the addition of 50 μL/well of TMB (Sigma-Aldrich). The reaction was terminated after 30 min by the addition of 25 μL/well of 0.5 M H_2SO_4, and absorbance values were determined at 450 nm using an ELISA microplate reader (iMark Microplate reader, Bio-Rad).

Mouse Spleen Cell Cultures and Determination of Cytokine Secretion. At the experimental end point, post oral challenge, spleens from individual mice were aseptically removed into ice-cold RPMI-1640 medium (Gibco Invitrogen, New York, NY), containing $NaHCO_3$ (1.5 g/L), glucose (4.5 g/L), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 U/mL), and streptomycin (50 mg/mL), and two whole spleens were pooled within each group (n = 5/group). The cell suspensions were passed through a 100 μm nylon membrane cell strainer, transferred to 15 mL conical centrifuge tubes, and centrifuged for 10 min at 500*g* at 4 °C. Erythrocytes in spleen cell preparations were lysed with 2 mL of red blood cell lysing buffer (Sigma-Aldrich), and 10 mL of RPMI medium was added to stop the lysis. The splenocytes were washed twice with 10 mL of RPMI by centrifugation. Splenocytes were resuspended in 10 mL of medium [RPMI 1640 supplemented with 8% fetal bovine serum (FBS)] (Hyclone, Fisher, Canada), and cell viability was assessed by trypan blue exclusion. Cells were cultured in 24-well plates (Corning) at a density of 2.5 × 10^6/mL in the absence (negative control wells) or presence of purified EW (100 μg/mL) in triplicates. Supernatants were collected after 72 h of incubation in a 5% CO_2 humidified incubator and assayed for the presence of cytokines. Concentrations of IFN-γ, IL-4, TGF-β, and IL-10 secreted in murine splenocyte culture supernatants were assayed by ELISA. Briefly, 96-well plates (Corning) were coated with 100 μL of the capture antibodies, rat anti-mouse IL-4 and INF-γ (BD) (1:250) or rat anti-mouse IL-4 (BD) (1:250), and the plates were incubated at 4 °C overnight. The plates were washed three times with 200 μL of PBST and blocked with 200 μL of 1% BSA in PBS at 37 °C for 1 h. The plates were further washed three times with PBST and 100 μL of standard cytokines (BD) at concentrations of 31.250−1000 pg/mL (IFN-γ) or 15.625−5000 pg/mL (IL-4) diluted in 1% (w/v) BSA in PBST, and the culture supernatant samples were added at different dilutions, 1:10 (IFN-γ) or 1:2 (IL-4), and incubated at 37 °C for 2 h. After four washings, 100 μL of detection antibodies was added, biotinylated rat anti-mouse INF-γ (BD) (1:2000) or biotinylated rat anti-mouse IL-4 (BD) (1:2000), and incubated for 1 h at 37 °C. The plates were washed four times, and bound antibodies were detected using 100 μL of HRP-conjugated avidin (BD) at 1:2000 dilution; plates

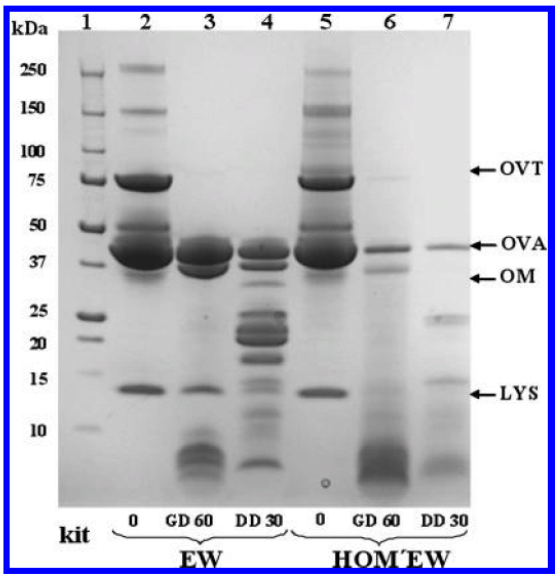

Figure 3. SDS-PAGE gel of in vitro gastric digestion at pH 2 (GD) and duodenal digestion (DD) of egg white (EW) and heated ovomucoid-depleted egg white (HOMEW) (40 μg/well). Lanes: 1, molecular marker; 2 and 3, GD of EW at 0 and 60 min, respectively; 4, DD of EW at 30 min; 5 and 6, GD of HOMEW at 0 and 60 min, respectively; 7, DD of HOMEW at 30 min.

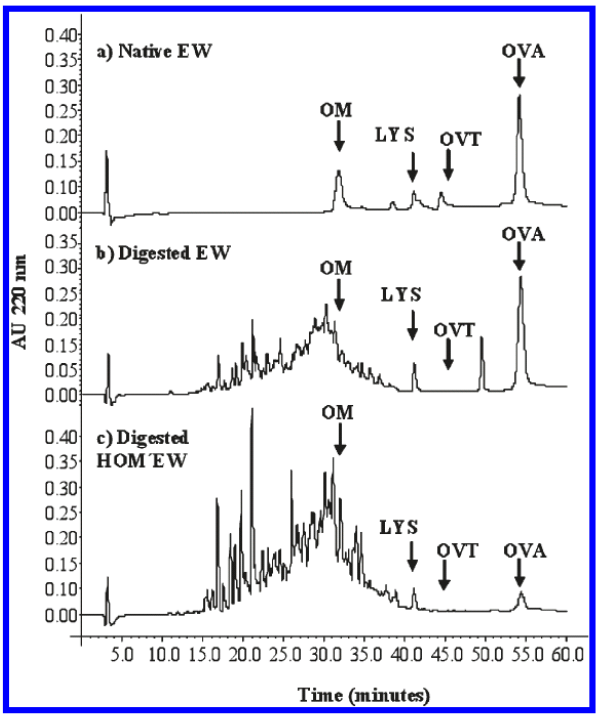

Figure 4. RP-HPLC analyses of nondigested egg white (EW) (a) and 60 min gastric digests of EW (b) and heated ovomucoid-depleted egg white (HOMEW) (c).

were then incubated for 30 min at 37 °C and washed six times with PBST. Then 50 μL of TMB (Sigma-Aldrich) was used as a substrate, the plate was incubated for 20 min in the dark at 37 °C, and 25 μL of stop solution (0.5 M H_2SO_4) was added. The optical density was measured by an ELISA reader (iMark Microplate reader, Bio-Rad) with a 450 nm filter. Production of TGF-β and IL-10 in the spleen cell culture supernatants was determined using the ready to use commercial kits TGF-B Ready-Set-Go and IL-10 Ready-Set-Go (eBiosciences Inc., San Diego, CA) following the manufacturer's instructions. Standard curves for each cytokine (15.625−1000 pg/mL TGF-β; 15.625−1000 pg/mL IL-10) were used to quantify the levels of cytokines present in the culture supernatant samples.

Statistical Analysis. Histamine, immunoglobulin, and cytokine concentrations measured by ELISAs were subjected to ANOVA analyses followed by post hoc multiple-comparison using Tukey's test. In all cases, P values of ≤ 0.05 were considered to be statistically significant. Statistical calculations were performed using the GraphPad Prism package (Graphpad, San Diego, CA).

RESULTS AND DISCUSSION

High in Vitro Digestibility of HOMEW. Digestibility of EW and HOMEW was performed to assess digestibility by using an in vitro system in two steps, which mimicked digestion in the stomach (gastric) and duodenum.[24] HOMEW was more susceptible to digestion than intact EW as shown in Figure 3 by SDS-PAGE. Looking at the 60 min gastric digests of EW and HOMEW (lanes 3 and 6), the band of OVA (44 kDa), the most abundant allergen in the albumen, is mainly intact in the EW, whereas is almost fully digested in the HOMEW, which presented a wide band of digestion products with molecular masses of <10 kDa. OVT (76 kDa) was fully digested in the gastric phase in both EW and HOMEW, whereas LYS (14.4 kDa) resisted the peptic action as part of the EW but not in the HOMEW. The higher digestibility of HOMEW was also kept during duodenal digestion (lanes 4 and 7), where the profile of HOMEW is clearer than that of EW because most of the protein content was completely hydrolyzed. Nondigested EW and 60 min gastric digests of EW and HOMEW were further analyzed by RP-HPLC (Figure 4), and the profiles were in agreement with SDS-PAGE outcomes. In the RP-HPLC profile of nondigested EW, the main EW allergens were identified as OVA that elutes at 55 min and OM after 33 min,[25] OVT at 46 min, and LYS at 42 min[26] (Figure 4A). A considerable amount of OVA was present in EW (Figure 4B), whereas it was almost undetectable in the 60 min gastric digest of HOMEW (Figure 4C). Upon peptic digestion the OM profile displayed a wide nonresolved mix of peaks that were difficult to identify within the complex matrix of digested EW (Figure 4B). It is interesting to note in the profile of the HOMEW gastric digest (Figure 4C) the increased amount of peptides from minutes 15−32 compared to that of the EW gastric digest (Figure 4B), which indicates the higher degree of hydrolysis in HOMEW. This increase in susceptibility to digestion of HOMEW can be attributed to the heat treatment because OVA is thermolabile and its digestibility increases by heat treatment.[25,27] LYS and OVT have also been reported to be unstable to heat treatment.[18] Furthermore, OM is reported to be digested into three fragments, and two of them resist duodenal digestion and are able to retain IgE binding activity;[10,25] hence, the low concentration of OM in HOMEW made it more susceptible to digestion. A high stability through digestion is usually accepted as a characteristic nature of a food allergen, which helps to keep the epitopes intact;[28] thus, the high digestibility of HOMEW may be related to the safety of OIT performed in this experiment. The gut-associated lymphoid tissues are widespread throughout the digestive tract in which the intestinal lamina propria contains a complex population of cells including activated CD4+ T lymphocytes and B lymphocytes, macrophages, dendritic cells, eosinophils, and mast cells. Also, there

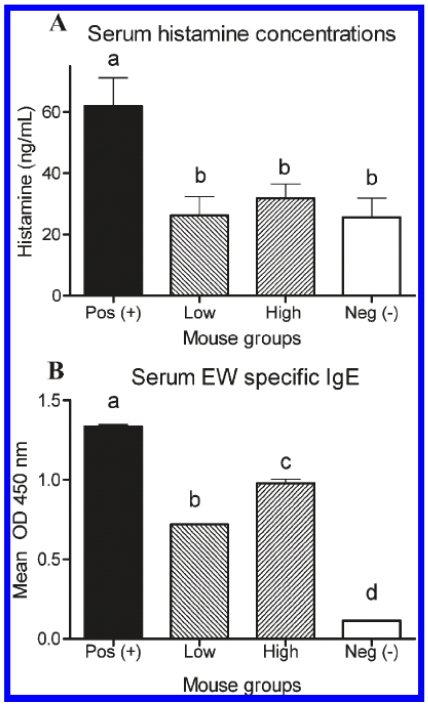

Figure 5. Serum histamine concentration and egg white-specific IgE activity in mice sera after oral challenge represented as mean standard deviation (n = 5) pooled sera. Different letters indicate statistically significant differences ($P < 0.05$) between groups of mice.

are organized lymphoid tissues, Peyer's patches being the most prominent, that likely have a pivotal role in triggering immune responses to digested antigens. Interestingly, it was recently reported that heat treatment of OVA and OM prevented transport across human intestinal epithelial cells in a form capable of triggering basophil activation or T-cell activation[29] and, together with the advanced degree of digestion of HOMEW at intestinal level, compared to intact EW, might be critical in the use of HOMEW for successful OIT. We recently reported oral administration of EW hydrolysates with peptic fragments of <1.4 kDa led to a specific immune hyporesponsiveness in EW-primed BALB/c mice.[30] It was also shown that the higher the digestibility, the lower the antibody binding, and heat treatment showed a significant influence on the potential allergenicity of the main egg white proteins that could be related to their resistance to denaturation and digestive enzymes.[25] Taken together, these data strongly support and augment our study in which HOMEW was able to induce successful tolerance to EW-sensitized mice, which may due to tolerogenic peptides present in HOMEW.

Low Histamine and EW-Specific IgE Activity after Oral Challenge. Histamine concentration was checked in mice sera after oral challenge with EW (Figure 5A). Both treated mouse groups had significantly less histamine than the positive group and were similar to the negative group, which confirmed the success of OIT. Also, EW-specific IgE antibody activity post oral challenge was less in both treatment groups than the positive group but significantly higher than negative group. Interestingly, the high-dose group showed higher EW-specific IgE activity than the low-dose group (Figure 5B).

Total and Allergen-Specific IgG and IgG Subclasses. Attempting to find other mechanisms that may have contributed to a lower histamine concentration, we studied in depth sera IgG activity. Total IgG and EW-specific IgG levels were similar in

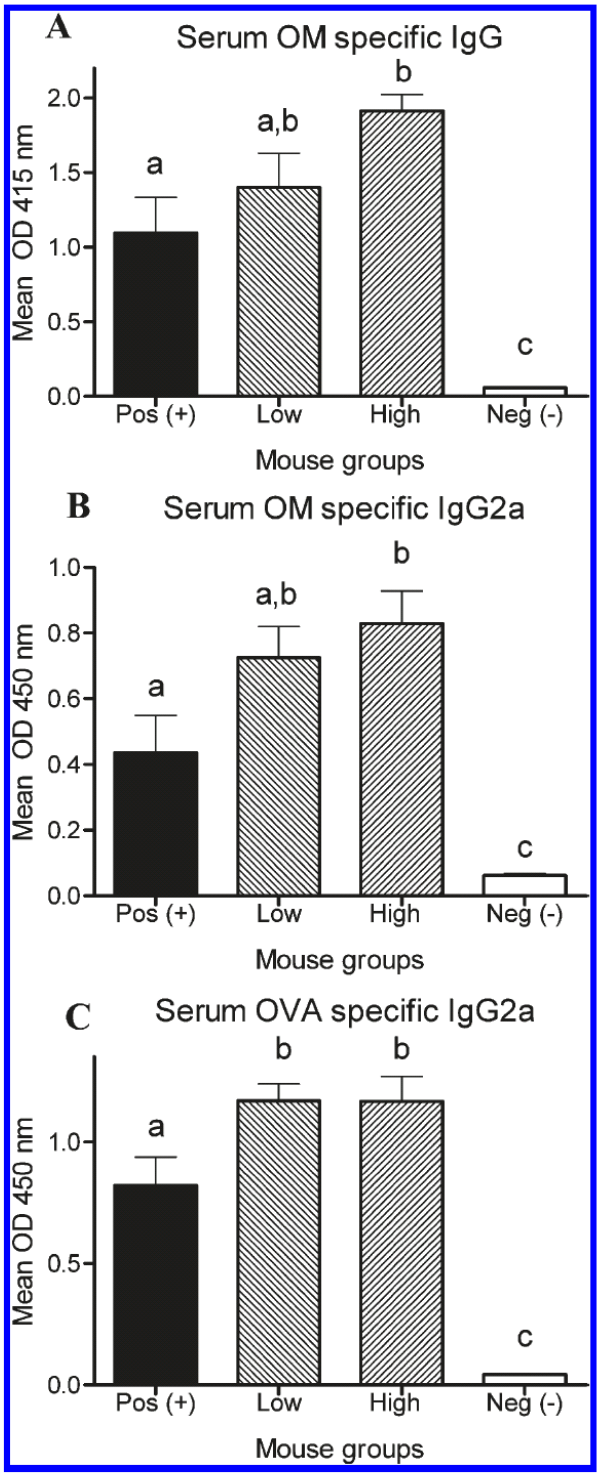

Figure 6. OM-specific IgG and IgG2a and OVA-specific IgG2a activities in mice sera post oral challenge. Data are represented as mean ± standard deviation (n = 5 pooled sera). Different letters indicate statistically significant differences ($P < 0.05$) between groups of mice.

sensitized mouse groups (data not shown). However, treated mice showed high OM specific IgG levels, and this increase was significant in the high-dose group (Figure 6A). Antibodies to specific IgG1 of EW, OVA, and OM were also analyzed without significant differences between groups; in contrast, EW-specific IgG2a was enhanced (data not shown) and OM- and OVA-specific IgG2a were significantly higher in the treated groups (Figure 6B,C).

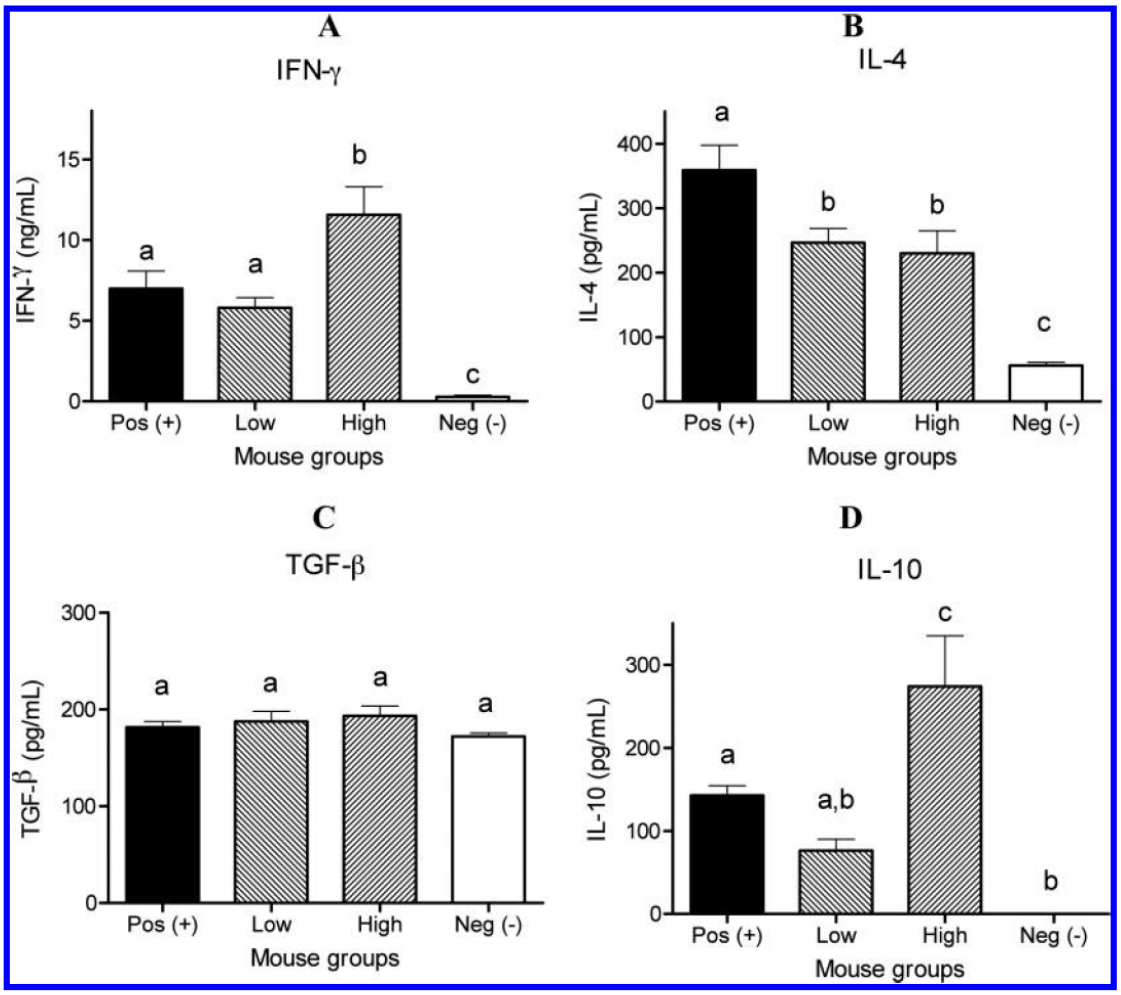

Figure 7. Determination of cytokine concentrations of IFN-γ, IL-4, TGF-β, and IL-10 following in vitro stimulation of spleen cell cultures with EW. Data are represented as mean ± standard deviation (n = 5 pooled spleens). Different letters indicate statistically significant differences ($P < 0.05$) between groups of mice.

The functions of allergen-specific IgG have been largely studied, but the roles in immunotherapy are still being investigated and are a point of discussion. One of the mechanisms related to allergen-specific immunotherapy is the induction of allergen-specific IgG antibodies, and it is suggested that these antibodies may compete with specific IgE to bind the allergen at the mucosal surface and block the allergic response. On the one hand, this is questioned because serum concentrations of allergen-specific IgG correlate with clinical improvement in some studies but not in others;[31] in addition, many mast cells are on the mucosal surfaces and could meet allergens before antibodies can interpose themselves.[32] On the other hand, it has been shown in a series of interesting experiments how allergen-specific IgG can counteract allergen-specific IgE activity by direct competition by binding epitopes or via inhibitory FcRIIB-dependent signals.[17] On this note, in the current study the increase of OVA- and OM-specific IgG2a and the increase of OM-specific IgG might be effective mechanisms induced during OIT that contribute to a lower histamine release.

Increased Th1/Th2 Ratio. The role of Th1 and Th2 cell mediated type responses is well established in mouse models, and recent research on T-regulatory cells sheds more light on the balance of Th1 and Th2 cell mediated response. In the present study, analyses of culture supernatants stimulated in vitro with purified EW indicated that the levels of IFN-γ, the type 1 hallmark cytokine, were significantly higher in the high-dose treated mice (Figure 7A), and no significant difference was observed in the low-dose treatment group as compared to the positive control. With regard to IL-4 concentration, the Th2 hallmark cytokine, the treatment groups (both high and low) had significantly less concentration as compared to the positive control group (Figure 7B). Also, concentrations of TGF-β (Th3) and IL-10 (Tr1) were analyzed, and no significant difference between groups was observed with TGF-β (Figure 7C); however, a significant increase of IL-10 concentration was found in the high-dose treatment group (Figure 7D), indicating a significant role of regulatory T cells in immune modulation caused by OIT with HOMEW.

It was of interest to note that the treatment effects were dose dependent and that the high-dose group experienced a marked increase in Th1/Th2 ratio, as a result of a significant increase of IFN-γ and a significant decrease of IL-4. The switch from Th2 to Th1 response could be attributed to a significant role of the regulatory cytokine IL-10. It has been reported earlier that in peanut-allergic patients undergoing OIT there is an increase in IL-10 secretion from peripheral mononuclear cells during the first months of OIT, which eventually tends to go down and likely depends on the stage of OIT: escalation, buildup, and maintenance.[33]

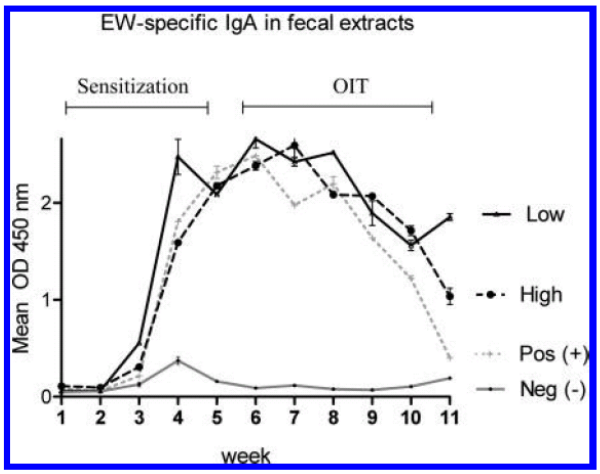

Figure 8. Time course monitoring of egg white (EW)-specific IgA levels in mouse fecal extracts before and during oral immunotherapy with heated ovomucoid depleted egg white. Data are represented as mean ± SD (n = 3).

This finding could be attributed to the low-dose group in which dose factor may play a significant role in OIT. In fact, it has been shown that IL-10 can modulate allergic reactions by different mechanisms: suppression of allergen-specific IgE, induction of allergen-specific IgG4/IgG2a, suppression of both allergen-specific Th1 and Th2 cells, and reduction of the release of pro-inflammatory cytokines by mast cells.[34−36] The suppression of antigen-specific immune responses by IL-10 is essential in peripheral tolerance to allergens. In mice it was shown to be the pivotal role of IL-10 in the establishment of peripheral T -cell tolerance by administration of IL-10, which drove mice to antigen-specific T-cell unresponsiveness.[37,38] Also, Enrique et al.[39] reported high levels of IL-10 in sera after sublingual immunotherapy in hazelnut-allergic patients. On the basis of our results, it may be postulated that IL-10 has exerted a critical role in immune modulation of the high-dose treatment group that might be indicative of a role of T-regulatory cells for induction of oral tolerance.

EW-Specific IgA in Fecal Samples. EW-specific IgA activity was determined in mouse fecal sample supernatants (Figure 8) in an effort to further elucidate the mechanisms occurring locally at the intestinal level. Both the allergic and treatment groups had an increase of EW-specific IgA throughout the sensitization phase. Once the sensitization phase had finished, the positive group experienced a decrease of EW-specific IgA, whereas treatment groups had an increase of EW-specific IgA at the beginning of OIT. Then specific EW−IgA activity decreased slowly but was always higher than the positive group activity. At the end of OIT, treatment groups showed higher activity of EW-specific IgA than the positive control group. The negative group had low activity of EW-specific IgA throughout the study. Secretory IgA has an important role in the immune homeostasis of the gut,[16] but its role in food allergy is still unclear. It has been earlier reported that low levels of allergen-specific IgA in the gut were associated with development of food allergies.[40] Although specific IgA was elevated during the sensitization phase, at the end of OIT, just before the end point, we can clearly see how the treated groups had a high activity of allergen-specific IgA, whereas the negative and positive groups had low activities of EW-specific IgA, suggesting a susceptible state to develop allergic disease, which supports previous studies[13,16,40] and reinforces the fact that a higher production of EW-specific IgA at the mucosal level may have contributed to the allergy-suppressive effect of HOMEW.

In conclusion, the present study establishes that the success of HOMEW can be related to its high digestibility. OIT with a high dose of HOMEW induced desensitization through a switch from Th2 to Th1 response marked by an increase in IL-10 concentration. Also, less histamine and EW-specific IgE and more specific IgG and IgG2a could have contributed to suppression of allergic response, and high amounts of EW-specific IgA in fecal samples suggest an important role at the mucosal level that might be contributing to the therapeutic effect of HOMEW.

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ABBREVIATIONS USED

HOMEW, heated ovomucoid-depleted egg white; OIT, oral immunotherapy; OVA, ovalbumin; OM, ovomucoid; LYS, lysozyme; OVT, ovotransferin; SGF, simulated gastric fluid; IL, interleukin; IFN-γ, interferon gamma; TGF-β, transforming growth factor beta; Th1, T-helper cell response type 1; Th2, T-helper cell response type 2; T-reg, T regulatory cell response.

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DISCUSIÓN GENERAL

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1. Digestión de la lisozima

La escasa bibliografia existente, y en ocasiones contradictoria, sobre la digestibilidad de la LYS y la alergenicidad remanente de los digeridos, nos llevó a estudiar el comportamiento de la LYS, bajo diferentes condiciones que imitasen la digestión fisiológica, lo cuál requirió de la puesta a punto y optimización en nuestro laboratorio de un sistema de digestión *in vitro* que simulase la digestión gástrica sucedida de la duodenal (Moreno y col., 2005; Martos y col., 2010). Con dicho modelo, se evaluó la influencia del pH, desde valores bajos, habituales en ayunas (1.2-2), a más elevados, que ocurren con el estómago lleno o son característicos en niños (3.2-4-5). La LYS se digirió únicamente a pH 1.2 y 2, dando lugar a fragmentos peptídicos menores de 6 kDa que resistieron la acción de la pepsina al menos durante 2 horas. La digestión fue mayor a pH 1.2, no pudiéndose detectar, por SDS-PAGE, LYS intacta tras 60 min de hidrólisis. El pH óptimo al que actúa la pepsina es amplio y se ha visto que, dentro del rango de pH en el que la pepsina es activa, su acción depende en gran medida de los cambios conformacionales que sufran las proteínas como consecuencia del pH (Schlamowitz y Peterson, 1959). A un pH estomacal muy ácido, típico de adultos en ayunas (1.2-2), la digestibilidad de la LYS se puede relacionar con el desdoblamiento de la LYS (De Laureto y col., 2002). Así pues, la pepsina, que requiere la unión y acoplamiento de secuencias extendidas de 8-10 aminoácidos con su centro activo para actuar (Fontana y col., 1986), podrá digerir mejor aquellas formas proteicas que se hallen desdobladas. En cambio, con una acidez menor, como la que se da en estados de saciedad o en los recién nacidos, se redujo el grado de hidrólisis de LYS, e incluso se impidió la digestión. El limitado grado de digestión de LYS a pH igual o superior a 3.2, podría ser de especial interés en bebés menores de 2 meses, cuyo pH estomacal es particularmente alto, ya que favorecería la llegada de LYS al duodeno, donde podría ejercer un papel antimicrobiano, pero también alcanzar el sistema inmune con facilidad debido a la mayor permeabilidad que caracteriza los estados de inmadurez intestinal (Vukavic, 1984).

Los resultados obtenidos pueden ayudar a comprender las discrepancias existentes sobre la resistencia de la LYS a la acción de la pepsina, que probablemente tengan su origen en la heterogeneidad de condiciones experimentales empleadas en los estudios, como son el pH del medio gástrico o la relación enzima:sustrato (E:S)—que

convendría expresar, para facilitar la comparación, en unidades de enzima por miligramo de proteína. Verbigracia, De Laureto y col. (2002) han descrito la gran estabilidad de LYS a la digestión pépsica empleando una relación E:S (p/p) de 1:500, a pH 2 y 20-20ºC; Ibrahim y col. (2005) publicaron que el 40% de la LYS se digiere tras 120 min de digestión a pH 4, empleando un ratio E:S (p/p) de 1:50, mientras que Mine y col. (2004) vieron que se digería completamente a pH 1 tras 60 min de hidrólisis empleando un ratio de E:S (p/p) de 1:25. Con estos precedentes parece necesaria la estandarización de los métodos de digestión *in vitro*, de modo que éstos reproduzcan condiciones relevantes fisiológicamente, para poder evaluar de forma fidedigna y reproducible el comportamiento de los alérgenos a través del sistema digestivo (Moreno, 2007). En términos metodológicos, el próximo reto que ha de afrontarse es el empleo digestores dinámicos, que además de un mayor grado de precisión, puedan reproducir mejor, desde un punto de vista físico, las transformaciones que sufre el alimento a través del tracto gastrointestinal (masticación, vaciado gástrico, peristaltis, etc.).

También se evaluó la influencia de surfactantes naturales, como la PC y las sales biliares, en diferentes concentraciones características de estados de ayuno y de saciedad, sobre la digestibilidad de LYS. La PC protegió levemente a la LYS de la acción de la pepsina, aunque no cambió el patrón de hidrólisis. Un comportamiento parejo se ha descrito para otras proteínas alimentarias, como la α-lactoalbúmina, dónde la mayor resistencia a la digestión en presencia de PC se explicó por la penetración parcial de la proteína en las vesículas de PC (Moreno y col., 2005). En cambio, la presencia de PC no afectó la digestión pépsica de otros alérgenos alimentarios como la albúmina 2 S de la nuez de Brasil (Moreno y col., 2005), la β-lactoglobulina (Macierzanka y col., 2009) o la OVA (Martos y col., 2010). El hecho de que la presencia de PC disminuyese la digestión de LYS era esperable, puesto que algunas de las propiedades biológicas de la LYS, como la antimicrobiana y la inmunomoduladora, se han atribuido a su capacidad de penetrar en las bicapas lipídicas (Gorbenko y col., 2007). En concordancia con tales resultados, vimos que, al mezclar la LYS con PC, aumentaba la hidrofobicidad de la LYS, posiblemente por la unión de ésta con la PC. Por tanto, la LYS podría interaccionar con fosfolípidos zwitteriónicos, como la PC, mediante interacciones polares o hidrófobas que produjesen la asociación de LYS con las vesículas de PC, de hecho, se ha descrito que la LYS podría quedar atrapada en liposomas no cargados de PC (Witoonsaridsilp y col., 2010).

El comportamiento de LYS en medio intestinal estuvo marcado por la presencia de sales biliares, que indujeron su precipitación, especialmente en condiciones experimentales que imitaban un estado intestinal de saciedad, que es cuando más cantidad de sales biliares se segrega (Kostewicz y col., 2002), aunque la PC evitó parcialmente la precipitación de LYS. Es probable que la LYS interaccione con las sales biliares mediante atracciones electrostáticas entre la proteína, fuertemente catiónica y las moléculas aniónicas de las sales. Por otra parte, de acuerdo con Gass y col. (2007), en presencia de PC o de otros lípidos, los ácidos biliares tienden a formar parte de micelas mixtas a nivel duodenal, lo que disminuiría la concentración de ácidos bililares libres capaces de interaccionar con las proteínas.

La precipitación de la LYS en condiciones de elevada concentración de sales biliares y bajo pH, podría explicar el hecho de que la administración oral de LYS con fines farmacológicos en humanos proporcione mayor absorción en ayunas, que cuando se toma junto con otros alimentos (Hashida y col., 2002). En cualquier caso, debe tenerse en cuenta que cuando se consume huevo entero, las lipoproteínas de baja densidad y la PC, presesentes en la yema, son capaces de unirse a las sales biliares, lo que dejaría, de modo natural, menos concentración disponible para inducir la agregación de la LYS. Experimentos realizados en nuestro laboratorio han puesto de manifiesto que cuando se simula la digestión gastrointestinal de huevo entero, la LYS permanece soluble bajo condiciones duodenales y resiste a la acción de las proteinasas pancreáticas (resultados no publicados).

Dado que la solubilidad de la LYS en condiciones duodenales se vio dramáticamente reducida, únicamente se midió la capacidad de unión a IgE de los digeridos gástricos de LYS. Para ello fue necesaria la optimización del método de ELISA de inhibición, también llamado de competición, con sueros de pacientes alérgicos, que fue compleja por el limitado volumen de sueros disponible y la baja señal que se detectaba, lo cual solucionamos incluyendo un paso de amplificación de la señal que mejoró ostensiblemente la detección y permitió un mejor aprovechamiento de los sueros al poder diluirlos. La ventaja principal de este método reside en preincubar la muestra problema (por ejemplo, un digerido gástrico de LYS) con sueros de pacientes alérgicos a huevo, antes de ser añadida a la placa, dándose un contacto íntimo entre los epítopos contenidos en la muestra y la IgE sérica, que evita la pérdida de pequeños fragmentos peptídicos que difícilmente se adherirían a la placa, estimándose con mayor precisión la alergenicidad *in vitro*. La otra ventaja de este tipo de ELISA es que, al hacer

diluciones seriadas de la muestra problema, se obtiene una curva sigmoidal dosis-respuesta, de pendiente variable, que permite calcular el IC50, es decir, la cantidad de proteína que une o inhibe el 50% de la IgE, y de este modo valorar la alergenicidad *in vitro* de las muestras que nos interesen.

Este sistema de ELISA reveló que durante la digestión gástrica de LYS, en presencia y ausencia de PC, se liberaban fragmentos proteicos con gran capacidad de unión a IgG e IgE equiparable o superior a la de la proteína nativa y que podrían ser responsables de su elevada alergenicidad. La unión a IgG e IgE fue mayor en los digeridos gástricos en presencia de PC, hecho que se puede achacar al efecto protector de la PC sobre la digestión de LYS, aunque tal resultado no fue significativo. Así pues, la precipitación de LYS en condiciones intestinales (intermedias y de saciedad) podría facilitar la llegada de la proteína a partes inferiores del intestino donde podría ejercer un papel defensivo, aunque la precipitación podría también afectar la cantidad de proteína inmunorreactiva intacta que podría ser absorbida.

2. Efecto del procesado sobre alérgenos de huevo

2.1 Ovoalbúmina

Durante el almacenamiento pueden ocurrir cambios estructurales en las proteínas, siendo este el caso de la OVA que, de modo natural durante el envejecimiento del huevo, se transforma en S-OVA y causa una merma en sus propiedades funcionales, no estando descrito cómo tales cambios afectan a su digestibilidad y potencial alergénico, cuyo estudio abordamos. Se observó que en las muestras de OVA y clara en las que se indujo la formación de S-OVA *in vitro*, la reactividad frente a IgE fue significativamente menor, hecho que en parte puede asociarse con algunos de los cambios estructurales descritos en la S-OVA y, en particular, con aquellos que afectan a Ser 164, Ser 320 y Phe 99, pues estos aminoácidos están presentes en regiones de la proteína en las que se han identificado epítopos (Kahlert y col., 1992). Además, el cambio conformacional que sufre Phe 99 induce una disminución en la accesibilidad al solvente de los aminoácidos adyacentes, entre los que se incluye Phe 378, un residuo que forma parte de un epítopo de la OVA localizado en una posición muy accesible en la superficie de la proteína (Honma y col., 1996). Por otra parte, el aumento de lámina β antiparalela en detrimento de hélice α que se da en la S-OVA (Kint y Tomimatsu, 1979) es indicativo de una desnaturalización parcial durante el tratamiento alcalino, que pudo haber contribuído a la pérdida de epítopos conformacionales.

Observamos, sin embargo, que la S-OVA era más resistente a la digestión que la OVA nativa, posiblemente porque los cambios anteriormente descritos la dotan de mayor estabilidad, solidez e hidrofobicidad (Huntington y Stain, 2001). La mayor resistencia de la S-OVA fue particularmente evidente durante la digestión gástrica simulada, mientras que las diferencias en cuanto a la susceptibilidad frente a la proteolisis de OVA y S-OVA se atenuaron durante la digestión duodenal. Por ello, y a pesar de las diferencias mencionadas, los hidrolizados gastroduodenales de S-OVA mostraron una baja reactividad frente a IgE, similar a la de la OVA. Esto puede deberse a la similitud entre los patrones de fragmentación de ambas y a que, dada la virtual desaparición de la proteína intacta, son los productos de digestión los principales responsables de la pequeña reactividad residual frente a IgE.

Es sabido que el tratamiento térmico provoca cambios fisicoquímicos en las proteínas dependendiendo de la naturaleza de éstas y de las condiciones del calentamiento (Wal, 2003). Los cambios fisicoquímicos inducidos en la OVA por el tratamiento térmico dieron lugar a resultados diferentes. El tratamiento térmico de la OVA afectó al patrón de separación de la proteína mediante SDS-PAGE, siendo tales cambios dependientes de la intensidad y duración del calentamiento. El calentamiento a 65ºC durante 30 min causó la formación de un fragmento de OVA de peso molecular ligeramente menor, coincidente con el fragmento de 40.1 kDa que se forma por ruptura del enlace His22-Ala23 por la pepsina (Martos y col., 2010), mientras que el calentamiento a 90ºC durante 15 min produjo, además de la aparición de tal fragmento, la formación de agregados de elevado peso molecular unidos por enlaces disulfuro. Asimismo, se ha descrito que la OVA calentada 3 min a 100ºC se fragmenta notablemente, detectándose por SDS-PAGE como una banda menor de 14kDa (Honma y col., 1994). A tal respecto, la OVA se ha descrito como una proteína termolábil; aunque existen ciertas discrepancias en cuanto a su temperatura de desnaturalización que, dependiendo del autor, puede ir desde 75 a 84ºC (Donovan y col., 1975; Hammershoj y col., 2002; de Groot y de Jongh, 2003). Es posible que la variabilidad encontrada en la bibliografía respecto a la temperatura de desnaturalización de la OVA se deba a la presencia inadvertida de S-OVA junto con la OVA nativa que, de acuerdo con estudios previos, es más estable al tratamiento térmico, hecho que pudimos corroborar en las muestras en las que inducimos formación de S-OVA *in vitro*, a partir de OVA y clara, con temperaturas de desnaturalización de 90.3 y 91.5ºC respectivamente.

Cuando se sobrepasó claramente la temperatura de desnaturalización de la OVA, con el tratamiento térmico a 90ºC durante 15 min, se redujo considerablemente su capacidad de unir IgE, hecho atribuible a la pérdida de epítopos conformacionales. Se ha descrito que, en condiciones más intensas, por ejemplo 3 min a 100ºC, incluso podrían perderse epítopos secuenciales debido a la intensa rotura de la proteína en la que, con seguridad, se ve afectada la estructura primaria (Honma y col., 1994). Otros estudios también han puesto de manifiesto que el calentamiento intenso de OVA reduce su capacidad de reconocer IgE, pero sin anularla por completo (Kim y col., 2002; Mine y Zhang, 2002). En cambio, cuando el tratamiento térmico no es intenso, la OVA puede pasar inalterada, verbigracia, al calentarse a 65ºC durante 30 min. Existe la posibilidad de que tratamientos térmicos menos intensos, que no desnaturalicen completamente la OVA, la lleven a un estado de "molten globule" en el que esté parcialmente desdoblada sin compromiso de la estructura terciaria (Campbell y col., 2003) y que podrían incluso aumentar su alergenicidad, ya que no habría una clara pérdida de epítopos conformacionales y se expondrían parte de los escondidos en la estructura. De hecho, cuando la OVA se desnaturaliza con urea o con ácido clorhídrico, no se detectan por SDS-PAGE cambios importantes en la estructura, pero la capacidad de unión a IgE es mayor que la de la proteína nativa (Honma y col., 1994).

La OVA sometida a calentamiento intenso (90ºC, 15 min) experimentó un aumento notable de su digestibilidad *in vitro,* mostrando un estado avanzado de digestión tras la fase gástrica que corrobora lo descrito por Takagi y col. (2003). Estos autores vieron un aumento de la hidrólisis de la OVA tras el calentamiento a 100ºC durante 5 min, aunque emplearon un modelo experimental que evaluaba de forma independiente la digestibilidad en condiciones gástricas y duodenales, en vez de hacerlo de forma sucesiva. La digestión gástrica de OVA calentada 90ºC y 15 min seguida de duodenal evidenció que la OVA quedaba totalmente hidrolizada tras la digestión duodenal, detectándose tan solo fragmentos peptídicos hidrofílicos y presumiblemente de bajo peso molecular por RP-HPLC. El calentamiento de la OVA aumentó su digestibilidad, posiblemente al facilitar el acceso de las enzimas digestivas a los puntos de corte específicos. La pérdida de epítopos conformacionales debida al tratamiento térmico, sumada a la de epítopos secuenciales por el avanzado grado de digestión, prácticamente abolió la capacidad de unión a IgE de la OVA calentada y digerida, lo que explicaría el hecho de que un 50% de pacientes alérgicos toleren la clara de huevo calentado (Urisu y col., 1997) donde la OVA supone el 54% del contenido proteico.

2.2 Ovomucoide

El efecto del tratamiento térmico del OM sobre su digestibilidad y alergenicidad *in vitro* fue motivo de estudio. El OM no experimentó cambios detectables por SDS-PAGE o RP-HPLC tras ser calentado debido a su gran termoestabilidad. Aunque la proteína, y cada uno de sus dominios, sufran desdoblamiento por el calor, se ha visto que tales cambios son reversibles (60-90ºC) (Matsuda y col., 1981). No obstante, el desplegamiento y posterior plegamiento de la proteína van a estar influenciados por diferentes factores como el pH y la fuerza iónica del medio, pudiendo resultar en que la renaturalización del OM no sea completa (Swintkruse y Robertson, 1995). En nuestro estudio, el calentamiento a 90ºC y 15 min redujo su capacidad de reconocimiento por IgE, posiblemente por pérdida de epítopos conformacionales, habiéndose descrito un resultado similar al calentarlo a 95ºC y 15 min (Mine y Zhang, 2002). También se ha sugerido que la renaturalización del OM tras ser tratado por calor (100ºC, 30 min) podría conducir a la formación de nuevos epítopos o, al menos, de epítopos específicos de OM desnaturalizado (Hirose y col., 2004).

La digestión gastrointestinal del OM calentado fue muy similar a la del nativo. Aunque frecuentemente se habla de la exigua digestibilidad del OM (Hirose y col., 2004), éste se degrada rápidamente con pepsina (Kovacs-Nolan y col., 2000). El hecho de que el OM nativo se digiera con tal prontitud en presencia de pepsina (en 10 min en nuestro estudio) podría dificultar la comparación de su digestibilidad con la del OM calentado. La resistencia del OM a la digestión se debe a los fragmentos formados durante la digestión gástrica que fueron visibles después de la duodenal. Tal resistencia, previamente descrita en el OM nativo, también se manifestó en el OM tratado térmicamente, sin hallarse diferencias entre ambos, y podría deberse a que el OM exhibe actividad inhibidora de la tripsina que, aunque se reduce durante la digestión gástrica, puede contribuir a mantener la integridad de sus fragmentos en el duodeno (Kovacs-Nolan y col., 2000), fragmentos que mantienen una reducida, pero perceptible, capacidad de unión a IgE.

El hecho de que el OM sea reconocido como el alérgeno inmunodominante del huevo es en gran parte debido a su resistencia al calentamiento, que apenas afecta a su estructura, ya que se renaturaliza en su mayoría, y a que éste no modifica su digestibilidad cómo ocurrió, por ejemplo, con la OVA. Así, la presencia de IgE reactiva frente a digeridos gástricos de OM se ha postulado como un indicador de alergia persistente en pacientes alérgicos a huevo (Urisu y col., 1999), aunque, puesto que el

entramado inmunológico del intestino va a ser determinante en el desarrollo de la alergia, quizá sería más apropiado determinar la reactividad de IgE frente a digeridos duodenales de OM como indicador de alergia persistente a huevo. Cuando testamos la unión a IgE de los digeridos duodenales de OM, vimos que estos fueron capaces de unir IgE y que, incluso los digeridos duodenales del OM calentado reconocieron IgE de forma similar a los de OM nativo, lo que evidencia el papel inmunodominante del OM en el huevo.

3. Efecto de la matriz sobre alérgenos de huevo

3.1 Interacción con glucosa por reacción de Maillard

Los diferentes resultados existentes acerca de la influencia de la RM sobre la digestibilidad y alergenicidad de proteínas alimentarias nos impulsó a estudiarla en los alérgenos principales de la clara (OVA y OM), cuyo contenido en glucosa contribuye a que se dé la reacción. Las medidas de color y grupos amino libres fueron indicadores del avance de la RM en la OVA y el OM. Los cambios inferidos en ambas proteínas por la RM fueron dependientes del tiempo de incubación, observándose los mayores cambios tras 96 horas de glicación. A ese tiempo, en ambas proteínas se produjo polimerización, mediante enlaces disulfuro, pero también mediante enlaces covalentes no reductibles, especialmente en el caso de la OVA, hecho que había sido previamente descrito (Kato y col., 1988). Además de la agregación, por SDS-PAGE también se vio un desplazamiento de las bandas de OVA y OM hacia valores ligeramente superiores de Mr, que indicaba la unión de moléculas de glucosa a las proteínas.

La RM modificó la capacidad de unión a IgE de OVA y OM de forma opuesta. En la primera, la alergenicidad *in vitro* disminuyó, lo que hace suponer que la OVA glicada sufrió cambios que conllevaron a la rotura de epítopos conformacionales, pudiendo el elevado grado de polimerización haber enmascarado epítopos en la superficie. Esta explicación para la reducción del reconocimiento de OVA glicada por anticuerpos se ha dado recientemente en el caso de proteínas de avellana modificadas por RM (Cucu y col., 2011). Tal disminución en la reactividad frente a IgE también ha sido descrita en el alérgeno Pru av 1 de la cereza tras ser glicosilado con glucosa o ribosa, atribuyéndose a cambios irreversibles en la estructura terciaria de la proteína, debidos a modificaciones en los aminoácidos nucleofílicos por interacciones con productos carbonílicos que causaron la pérdida de epítopos conformacionales (Gruber y col., 2004).

En el caso del OM, la RM aumentó la capacidad de unión a IgE respecto del OM nativo, hecho que se explicaría por la formación de nuevos epítopos o porque se favoreció el reconocimiento de los ya existentes. De forma semejante, en los alérgenos Ara h 1 y Ara h 2 del cacahuete se ha descrito un aumento de la unión a IgE tras RM con diferentes carbohidratos, entre los que estaba la glucosa, explicándose tal aumento por la formación de nuevos puntos de unión a IgE, la exposición de epítopos protegidos en la estructura e, inclusive, por la formación de productos avanzados de la RM capaces de ser reconocidos por IgE (Maleki y col., 2000).

Un aspecto controvertido y común en los estudios revisados, incluido el presente, es el repertorio de IgE específico de cada paciente. Verbigracia, en nuestro estudio, los sueros empleados provienen de pacientes alérgicos a huevo, de los que se conocen las titulaciones frente a clara, yema, OVA y OM, pero no así la especificidad de cada IgE—los epítopos del alérgeno en cuestión que cada paciente reconoce—, es decir, cómo se ha sensibilizado y frente a que forma de OVA y OM. Los sueros estudiados reconocieron el OM glicado con más facilidad que el nativo, pudiendo hacerse conjeturas dependiendo de si ha habido o no una exposición previa del paciente a OM glicado. Si no ha habido tal exposición, se podría pensar que el OM glicado ha podido desdoblarse parcialmente y exponer epítopos a los que el paciente estaba previamente sensibilizado, o bien que se hayan formado nuevos epítopos que guardan suficiente similitud con los ya existentes. Si ha habido una exposición previa a OM glicosilado, el paciente se ha podido sensibilizar realmente frente a los nuevos epítopos, no existentes en el OM nativo, lo que explica, por ejemplo, que se hayan dado casos de reacciones anafilácticas en pacientes sensibilizados únicamente frente a neoepítopos formados por el procesado y no frente al alérgeno en su forma nativa (Malanin y col., 1995). A tal respecto, en una investigación actual en ratones, se ha descrito que la OVA glicada es más inmunogénica que la nativa ya que la primera se reconoce mejor por células T (Ilchmann y col., 2010).

Nuestros resultados apoyan estudios anteriores sobre la influencia de la RM sobre la capacidad de unión a IgE de algunos alérgenos alimentarios, que han proporcionado resultados diferentes en función del tipo de proteína, del azúcar y de las condiciones de reacción. No obstante son muy escasos los estudios sobre la digestibilidad de alérgenos glicados y más aún aquellos que evalúan el reconocimiento por IgE de los digeridos gastroduodenales, a pesar de la relevancia de los últimos en la patogénesis de la alergia a alimentos.

En nuestro estudio, evaluamos tanto la digestibilidad de la OVA y el OM glicados como la capacidad de unión a IgE de sus digeridos duodenales. La OVA glicada fue más resistente al proceso digestivo que la nativa, puesto que, tras las digestión gástrica seguida de la fase duodenal, todavía se detectó OVA glicada intacta y agregados de elevado peso molecular. Probablemente, la agregación causada por RM dificultó la acción de las enzimas digestivas protegiendo así a la proteína de la hidrólisis. El bloqueo de los residuos de Arg y Lys, sobre los que actúan tripsina y quimotripsina, puede haber contribuido a la menor digestibilidad de la OVA glicada. En otros estudios, se ha visto que la RM reduce la digestibilidad de diferentes alérgenos alimentarios y, aunque en muchos de ellos tan solo se evaluó ésta en condiciones gástricas (Maleki y col., 2000; Nakamura y col., 2006), también se ha demostrado tras digestión gastroduodenal *in vitro*, como en el caso de las proteínas de trigo (Simonato y col., 2001) o de la β-lactoglobulina de la leche (Sanz y col., 2007), habiéndose asociado tal resistencia con la polimerización de la proteína. Cuando se evaluó la capacidad de unión de los digeridos duodenales de OVA glicada, estos mostraron una reactividad similar a los de la nativa, lo que indica que la reducción inicial de la capacidad de unir IgE que se dio en la OVA glicada respecto de la nativa se vio contrarestada al dificultarse la digestión, resultando en una menor pérdida de epítopos. En algunos casos, como es el de la tropomiosina de calamar, aunque haya habido una reducción inicial de la capacidad de unión a IgE de la proteína glicada, la disminución de la digestibilidad, tras ser glicosilado con ribosa, fue tan acentuada que los digeridos resultantes de la proteína glicada fueron más reactivos que los de la nativa (Nakamura y col., 2006), mas no se llegó a evaluar la reactividad de los digeridos duodenales, lo que hubiera hecho el estudio más interesante. Al OM no lo afectó la RM en términos de digestibilidad siguiendo un patrón de digestión similar al del OM nativo. En comparación con la OVA glicada, la polimerización en el OM glicado fue menor, pudiendo no ser suficiente como para ejercer un efecto protector, de hecho, los agregados formados por RM se digirieron rápidamente durante la digestión gástrica. Por otra parte, aunque la RM pudo haber creado neoepítopos en el OM, estos sufrieron un grado de digestión similar al de la nativa, dando digeridos duodenales con similar capacidad de unión a IgE.

3.2 Interacciones no covalentes con polisacáridos

Los alimentos contienen proteínas y polisacáridos, que podrían interaccionar formando biopolímeros mixtos, tanto en el propio alimento, como durante la digestión fisiológica. La investigación del comportamiento durante la digestión gastrointestinal *in vitro* de mezclas de OVA u OM con diferentes polisacáridos de uso común en la industria alimentaria (P, G y X), y de su unión a IgE, puso de manifiesto una reducción de la digestibilidad de la OVA y OM en presencia de los polisacáridos. El hecho de que la OVA nativa se digiriese menos en presencia de P, G y X sugiere que ésta pudo interaccionar débilmente con los polisacáridos, especialmente en las condiciones ácidas de la digestión gástrica, y que tales interacciones dificultaron la hidrólisis de la proteína. En el caso del OM, que se hidroliza rápidamente con pepsina, tal efecto protector se vio reflejado en la digestión duodenal. A pH ácido, tanto en los propios alimentos como en el estómago, las proteínas, por debajo de su pI, pueden establecer asociaciones electrostáticas con polisacáridos aniónicos, como G y P, así como atracciones no iónicas –hidrofóbicas o mediante puentes de hidrógeno- que refuerzan las anteriores o son responsables de las uniones con polisacáridos no aniónicos, como X (Mackie y Macierzanka, 2010). A pH neutro, típico del duodeno, todavía pueden darse interacciones electrostáticas entre los polisacáridos aniónicos y las regiones cargadas positivamente de las proteínas, que también se intensifican mediante atracciones no iónicas (Dickinson 1998). Tales asociaciones podrían ejercer un efecto protector sobre la digestión de las proteínas, como se ha encontrado en estudios en los que se ha investigado el efecto de la presencia de P, G y X en la digestión *in vitro* de proteínas de cacahuete (Mouecoucou y col., 2004) y β-lactoglobulina (Mouecoucou y col., 2003). .

En nuestro caso, la mayor protección se dio en presencia de P, cuya capacidad de formar geles, especialmente en condiciones ácidas, podría haber ralentizado el avance de la digestión como se ha visto, tanto *in vivo* como *in vitro*, en el alérgeno Act c 2 del kiwi (Polovic y col., 2007). Aunque tal capacidad se ve perjudicada por las condiciones intestinales, se ha descrito que los geles de P, formados en condiciones gástricas, podrían resistir lo suficiente en el duodeno como para ejercer un efecto protector sobre la proteína en términos de digestibilidad (Polovic y col., 2009).

Las pruebas de ELISA de inhibición, una vez descartada la unión a IgE de G, P y X, mostraron que los digeridos duodenales de OVA y OM obtenidos en presencia de polisacáridos tuvieron mayor capacidad de unir IgE que los controles de OVA y OM digeridos en ausencia de polisacáridos. Por una parte, ésta mayor alergenicidad *in vitro*

podría atribuirse al menor grado de digestión que se dio en tales muestras, sin embargo, las diferencias observadas en cuanto a digestibilidad parecían demasiado pequeñas para justificar que la reactividad frente a IgE fuese significativamente mayor. Por otra parte, se ha sugerido que durante la digestión de las proteínas en presencia de polisacáridos, estos interaccionan con los productos de digestión en mayor medida que con la proteína intacta (Mouecoucou y col., 2003, 2004) y, de hecho, los análisis mediante SEC pusieron de manifiesto la posible interacción de los productos de digestión gástricos y duodenales de la OVA y el OM con los distintos polisacáridos.

Basándonos en estos resultados, se podría pensar que los fragmentos peptídicos producidos por la pepsina durante la digestión gástrica simulada podrían haber interaccionado electrostáticamente con P y G, resistiendo parcialmente, al menos, la hidrólisis duodenal posterior. Asimismo, los péptidos conteniendo aminoácidos básicos como Arg o Lys, liberados por la acción de la tripsina, también podrían interaccionar electrostáticamente con los polisacáridos aniónicos como P o G, mientras que con X se darían interacciones no iónicas. Si bien se ha indicado que la interación de polisacáridos con digeridos gástricos o duodenales reduce la unión a IgE, lo que se ha atribuído a un enmascaramiento de los epítopos por parte del polisacárido (Mouecoucou y col., 2003, 2004), también es razonable suponer que los complejos formados entre polisacáridos y fragmentos peptídicos hayan contribuido a conservar los epítopos.

A tal respecto, las fracciones de elevado peso molecular presentes en los digeridos duodenales de OVA y OM fueron recolectadas tras el análisis mediante SEC y su capacidad de unir IgE fue evaluada cualitativamente, mostrando que todas las fracciones, aunque con diferente intensidad, poseían reactividad frente a IgE. Debe destacarse que se han encontrado, entre los diferentes carbohidratos unidos a proteínas, algunos que pueden dar reacciones cruzadas con determinados anticuerpos IgE y que se denominan determinantes antigénicos de carbohidratos (CCD, Commins y Platts-Millls, 2010). En particular, se ha visto que los residuos de β 1,2-xilosa reaccionan *in vitro* con IgE específicas de alergia al polen y a alimentos (Kaulfüst-Soboll y col., 2011), lo que podría explicar la elevada reactividad frente a IgE encontrada en nuestro estudio.

Estos resultados destacan la importancia de la matriz del alimento en la digestibilidad de los alérgenos y en su capacidad potencial para desencadenar una respuesta inmune. Además, el hecho de que los polisacáridos puedan proteger a las proteínas o a sus productos de degradación de la acción de las enzimas digestivas, manteniendo su potencial alergénico, podría ser de especial importancia teniendo en

cuenta que la absorción de proteínas en presencia de polisacáridos aniónicos puede estar modulada (Schmidgall y Hensel, 2002), como sucede con la P, que es capaz de adherirse al mucus de la mucosa intestinal permitiendo mayor contacto de las moléculas unidas a P y el epitelio intestinal (Liu y col., 2005) con el subsiguiente efecto en la capacidad de desencadenar una respuesta alérgica.

3.3 Interacción con lípidos

La escasa bibliografía existente sobre el comportamiento de alérgenos de huevo en emulsiones alimentarias, referido a digestibilidad y alergenicidad, motivó su estudio. Para tal menester se preparó una emulsión O/W, a base de huevo liofilizado y aceite de oliva purificado, cuya estabilidad y características fueron evaluadas. La monitorización de la conductividad, sin apenas variación durante las primeras 48 horas, permitió definir el periodo en el que la emulsión elaborada conservaba las características iniciales. El estudio de la microestructura de la emulsión recién formada reveló la presencia de pequeñas gotas de aceite, con una capa interfacial alrededor, homogéneamente dispersas en la disolución proteica. Después de 4 horas no se observaron cambios en la microestructura, especialmente aquellos que son indicadores de desestabilización, como la floculación o coalescencia (McClements, 1999), procediéndose a estudiar en ese intervalo de tiempo la digestibilidad *in vitro* de la emulsión.

La digestión gastroduodenal *in vitro* de la emulsión no mostró diferencias respecto del control de huevo en cuanto al patrón de digestión, mas sí se vio ligeramente favorecida la hidrólisis en la emulsión. Otras proteínas alimentarias han mostrado mucha mayor susceptibilidad a la hidrólisis al estar emulsionadas, tal es el caso de la β-caseína y la β-lactoglobulina de la leche, cuya digestión gástrica es 2 y 10 veces más rápida, respectivamente, al formar parte de una emulsión O/W con aceite de oliva, siendo relacionados tales cambios con el desdoblamiento de las proteínas en su interacción con la interfase (Macierzanka y col., 2009). De hecho, es sabido que la β-lactoglobulina se desdobla con facilidad en su adsorción a la interfase durante la emulsificación (Husband y col., 2001), lo que puede ser responsable del aumento en su digestibilidad, ya que tales estados de desdoblamiento favorecen el acoplamiento de la pepsina a la cadena polipeptídica que va a hidrolizar (Kageyama, 2002). En la emulsión de huevo sólo se favoreció ligeramente la digestión de algunas proteínas entre las que destacó la OVA, por ser la mayoritaria en la clara. Esto indica que, en el caso de las proteínas de clara de huevo no tienen lugar cambios inducidos por la

adsorción que aumenten de modo importante su flexibilidad o susceptibilidad a la pepsina, probablemente porque las proteínas más resistentes a la acción de la pepsina, OVA y LYS, poseen una gran estabilidad conformacional a pH 2.0 (de Laureto y col., 2002; Tatsumi y col., 1999). De hecho, ni OVA ni LYS son buenas emulsionantes, y son los fosfolípidos de la yema los components que más contribuyen a esta propiedad en el huevo (Mine y col., 1992). Posteriormente, en el medio duodenal, los ácidos biliares, desplazarían a las proteínas que se hubieran adsorbido, por lo que la hidrólisis por enzimas pancreáticas tendría lugar sobre todo en disolución (Macierzanka y col., 2009).

La capacidad de unión a IgE del huevo emulsionado no fue significativamente distinta a la del huevo control. En cambio, sí se vieron diferencias en la reactividad frente a IgE entre los digeridos gástricos y duodenales de huevo y huevo emulsionado. Estos últimos presentaron menor capacidad de unir IgE que los digeridos de huevo, hecho que se atribuyó al mayor grado de hidrólisis que se vio en la emulsión, gracias al cual se pudo dar mayor pérdida de epítopos durante la digestión, con la subsiguiente reducción del potencial alergénico. Esta observación pone de nuevo de manifiesto la importancia de la resistencia a la digestión en los alérgenos alimentarios (Astwood y col., 1996), aunque tampoco podría descartarse cierto enmascaramiento de epítopos en su adsorción a la interfase.

4. Efecto inmunomodulador de fórmulas de huevo procesado

En un intento de esclarecer los mecanismos subyacentes de la OIT se estudió el efecto inmunomodulador de clara de huevo calentada con bajo contenido de OM en ratones Balb/c alérgicos a huevo. En primer lugar, se evaluó la digestibilidad *in vitro* de la fórmula de clara de huevo calentada con bajo contenido en OM, encontrándose una elevada susceptibilidad a la hidrólisis comparada con clara de huevo, que es probablemente responsable del carácter hipoalergénico de la fórmula. Inicialmente, el tratamiento térmico afectaría a la capacidad de unión a IgE de la OVA, como vimos anteriormente y, presumiblemente a la de otros alérgenos mayoritarios de la clara que son termolábiles, como LYS y OT, con la subsiguiente reducción del potencial alergénico. Además, el tratamiento térmico favorece claramente la digestión de tales alérgenos, lo que contribuye a acelerar la destrucción de epítopos durante el proceso digestivo. Dado que el OM es termoestable, y genera durante la digestión gástrica

fragmentos resistentes a la digestión duodenal con actividad inhibidora de tripsina y que contienen epítopos de unión a IgE, al reducir su contenido en la fórmula de inmunoterapia se mejora la digestibilidad y se contribuye a que esta sea hipoalergénica. La seguridad de la fórmula de OIT es requisito deseable mas no imprescindible, de hecho en muchos protocolos de OIT se usa el alimento ofensivo tal cual, si bien en cantidades que no producen sintomatología clínica (Buchanan y col., 2007; Vickery y col., 2010). No obstante, la capacidad terapéutica es un requisito imprescindible en los preparados de OIT y ésta podría estar relacionada con una elevada digestibilidad, puesto que durante la digestión gastrointestinal se liberan péptidos con capacidad inmunomoduladora que son reconocidos por células T. En esta misma línea, se ha visto como un hidrolizado de trigo que fue administrado a pacientes alérgicos tuvo capacidad de desensibilizar sin que hubiera peligro durante el tratamiento por ser hipoalergénico (Tanabe, 2007). También se ha descrito el poder terapéutico en ratones de una mezcla de péptidos del alérgeno del cacahuete Ara h 2 (Li y col., 2001) y, recientemente, se ha comprobado la capacidad de desensibilizar ratones alérgicos a huevo con fragmentos peptídicos menores de 1.4 kDa procedentes de un hidrolizado de clara de huevo (Yang y col., 2009).

La OIT resultó provechosa, a la vista de la concentración de histamina en sangre tras la provocación, significativamente menor en los grupos tratados. Los niveles de IgE también fueron significativamente inferiores en los ratones tratados que en los alérgicos, aunque, en los primeros, la IgE específica frente a clara fue mayor que en el control negativo, lo cual avala que no siempre niveles relativamente elevados de IgE específica van a desembocar en una respuesta alérgica (Urisu y col., 1999), pues probablemente haya mecanismos que atenúen la misma.. A tales mecanismos podrían contribuir las distintas subclases de IgG. Así, el claro aumento de IgG e IgG2a específicas tras la OIT podría aminorar la respuesta alérgica, ya sea por una competición directa frente a IgE, por unirse a los epítopos, o de manera independiente, enviando señales de inactivación a los mastocitos a través de los receptores FcγRIIB (Uermosi y col., 2010). Además, también se ha encontrado en estudios clínicos de OIT un incremento de la IgG2a, equivalente a la IgG4 en humanos (Mousallem y Burks, 2012), lo que refuerza su utilizad como biomarcador para monitorizar la progresión del tratamiento.

La estimulación de esplenocitos con clara de huevo y el posterior análisis de la producción de citoquinas mostró un claro aumento de la respuesta Th1 sobre la Th2,

que podría atribuirse a una elevada producción de IL-10. En otras investigaciones sobre la OIT se han descrito niveles altos de IL-10 de forma pasajera durante la inmunoterapia, coincidiendo con el cambio a una respuesta predominantemente Th1 (Vickery y col., 2010). La diferencia en los niveles de IL-10 determinados en los grupos de ratones tratados puso de manifiesto la importancia de la dosis en los efectos de la OIT. Tanto la dosis alta como baja de consiguieron reducer la respuesta alérgica, sin embargo, los cambios inmunológicos se vieron afectados por la dosis empleada, siendo más pronunciados en los ratones tratados con mayor cantidad.

La monitorización semanal de los niveles de IgA específicos en heces a lo largo del estudio, plantea cuestiones de interés. Los niveles bajos de IgA específica en el intestino pueden ser indicadores de un estado susceptible de desarrollar alergia (Frossard y col., 2004), pero no siempre los niveles altos van a ser un marcador fiable de tolerancia. De hecho, se han descrito casos de ratones sensibilizados que presentaban niveles altos de IgA especifica en heces (Lee y col., 2001). Se piensa que tanto los ratones alérgicos como los tolerantes tienen capacidad de producir IgA mediante las poblaciones celulares presentes en las placas de Peyer, aunque tal producción va a ser mayor en ratones tolerantes (Frossard y col., 2004). De acuerdo con tales resultados, al final de la OIT se comprobó que los grupos de OIT presentaron mayores concentraciones de IgA específica en heces que el positivo o negativo, pudiendo ser un indicador favorable. No obstante, conviene una interpretación cuidadosa de los niveles de IgA específicos. Los niveles elevados de IgA específica en suero se relacionan con sensibilización a alimentos, aunque se ha especulado que tal incremento es debido a que, en individuos tolerantes, la producción de IgA se da mayoritariamente en las placas de Peyer, permitiendo liberar elevadas cantidades de la misma en el intestino, mientras que en alérgicos se genera fundamentalmente en nódulos linfáticos mesentéricos con el subsiguiente aumento de IgA a nivel sérico (Frossard y col., 2004). Por tanto, la inclusión del ratio de niveles específicos de IgA intestinal / IgA sérica como marcador del estado del individuo alérgico podría ser útil, valorado junto con el resto de marcadores habituales.

Por último, un aspecto de gran importancia es establecer el grado de protección proporcionado por la OIT y determinar si se ha conseguido alcanzar la tolerancia o tan solo desensibilización. Ello requeriría llevar a cabo estudios en los que se incluyan grupos adicionales de ratones a los que, una vez terminada la OIT, se administrarían distintas dosis de mantenimiento para ver la posibilidad de recaer o no a largo plazo, e

identificar biomarcadores que sirvan como indicadores de un estatus seguro o de riesgo. No obstante, aun en aquellos casos en los que no sea posible inducir tolerancia oral, las mejoras alcanzadas por la OIT relatadas en el presente estudio y en la bibliografía (Mousallem y Burks, 2012) la hacen más adecuada que la evitación total del huevo, como forma de encarar la alergia.

CONCLUSIONES

CONCLUSIONES

1. Distintas condiciones de hidrólisis que imitan estados de saciedad y ayuno afectan a la digestión gástrica de la LYS, fundamentalmente por la influencia del pH y por la presencia de fosfatidilcolina, que la protege ligeramente de la hidrólisis. Los fragmentos proteicos liberados durante la digestión gástrica *in vitro* de la LYS muestran una notable capacidad de unión a IgG e IgE.

2. La LYS precipita en condiciones intestinales que simulan estados prandiales y posprandiales. La fosfatidilcolina evita parcialmente la precipitación de la LYS por acción de las sales biliares. Este comportamiento puede afectar al papel defensivo y a la alergenicidad de la proteína.

3. La S-OVA posee menor reactividad frente a IgE que la OVA, probablemente por la modificación que sufren algunos aminoácidos contenidos en regiones de la proteína nativa identificadas como epítopos. La S-OVA es más resistente a la digestión gástrica *in vitro* que la forma nativa, pero esta diferencia se atenúa durante la digestión duodenal, por lo que los hidrolizados de ambas presentan similar reactividad frente a IgE.

4. El tratamiento térmico reduce la capacidad de unión a IgE de la OVA, lo que, junto con el aumento de la digestibilidad que produce, anula prácticamente la capacidad de unión a IgE de sus digeridos duodenales. El tratamiento térmico también disminuye la unión a IgE del OM, pero no afecta a su digestibilidad, ni a la reactividad frente a IgE de sus digeridos duodenales.

5. La reacción de Maillard con glucosa disminuye la capacidad de unión a IgE de la OVA, aunque también reduce su digestibilidad, por lo que los digeridos duodenales de la OVA glicada muestran una unión a IgE similar a los de la nativa.

6. La reacción de Maillard con glucosa aumenta la capacidad de unión a IgE del OM, pero no afecta a su digestibilidad, ni a la unión a IgE de sus digeridos duodenales.

7. La presencia de pectina, goma arábiga y xilano dificultan en distinta medida la digestión de OVA y OM, ya sea al interaccionar con la proteína nativa o con fragmentos peptídicos resultantes de su hidrólisis. La mezcla con polisacáridos aumenta la reactividad frente a IgE de las proteínas y de sus digeridos gastroduodenales, siendo el efecto dependiente del tipo de proteína y polisacárido.

8. La digestibilidad *in vitro* de proteínas de huevo formando parte de una emulsión estable de aceite en agua es ligeramente mayor que la de las proteínas de huevo no emulsionadas. Tal variación en la digestibilidad afecta a la unión a IgE de los digeridos duodenales de la emulsión, que es menor que la de los digeridos de huevo en solución.

9. La hipoalergenicidad y las propiedades terapéuticas de la clara de huevo calentada con bajo contenido en OM probablemente residan en su elevada digestibilidad, inducida por el procesado. La inmunoterapia oral con dicha fórmula en ratones impide reacciones alérgicas manifiestas tras una prueba de provocación oral, disminuyendo la liberación de histamina y la concentración sérica de IgE específica.

10. La inmunoterapia oral produce un incremento del balance Th1/Th2, al reducir el nivel de IL-4 y aumentar el de IFN-γ, en parte regulado por la IL-10, y causa un aumento de los niveles de IgG e Ig2a específicas que podrían atenuar la respuesta alérgica. Además, induce mayores niveles de IgA específica en el intestino que podrían ejercer una función beneficiosa a nivel de la mucosa intestinal.

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